Universidad Autónoma de Madrid. Facultad de Ciencias. Departamento de Biología Molecular.
EFFECT OF THE INHIBITION OF THE PHOSPHATASE CALCINEURIN IN A MURINE MODEL OF EXPERIMENTAL ATHEROSCLEROSIS.

Tesis Doctoral
Maria Torrente Regidor.
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MEMORIA DE TESIS DOCTORAL

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Presentada por

Maria Torrente Regidor

para la obtención del grado de doctor por la

Universidad Autónoma de Madrid

Dirigida por:

Dr Antonio Rodríguez Márquez

“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”
Marie Curie

“We know what we are, but know not what we may be.”
William Shakespeare

“I've got to admit it's getting better. It's a little better all the time.”
Paul McCartney, The Beatles

Para mis padres.

Para mi hermano.

Para Dani.

Agradecimientos

Esta tesis no podría haberse realizado sin el trabajo, esfuerzo y colaboración de muchas personas, tanto de mi ámbito laboral como del personal. En mi caso concretamente éste podría llegar a ser uno de los apartados más extensos de este libro.

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Abbreviations

ABBREVIATIONS

AAV	Adeno-associated virus
AID	Autoinhibitory domain
apoE	Apolipoprotein E
BBD	CnB-binding domain
CaM	Calmodulin
CN	Calcineurin (Heterodimer: CnA bound to CnB)
CnA	Calcineurin subunit A
CnB	Calcineurin subunit B
CsA	Cyclosporine A
CVD	Cardiovascular disease
Cyp	Cyclophilin
DKO	Double knock out
EC	Endothelial cell
ESELp	E-selectin promoter
FKBP	FK506-binding protein
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
HUVEC	Human umbilical vein endothelial cells
i.p.	Intraperitoneal
i.v.	Intravenous
ICAM-1	Intracellular adhesión molecule-1
IFN-γ	Interferon gamma
IL	Interleukin
iMLEC	Immortalized mouse lung endothelial cells
IPs	Immunophilins
IS	Immunosuppressants
KO	Knock out
LDL	Low density lipoprotein
LDLR-KO	LDL receptor-knockout
LPS	Lipopolysaccharide
LV	Lentiviral vector
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage colony stimulating factor
MEF-2	Myocyte enhancer factor-2
MLEC	Mouse lung endothelial cells
MMP	Matrix metalloproteinase
ND	Normal diet
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ox-LDL	Oxidized low density lipoproteins
PDGF	Platelet-derived growth factor
PMA	Phorbol myristate acetate
RA	Rheumatoid arthritis
SFFVp	Spleen forming focus virus promoter
SMC	Smooth muscle cells
TGF	Transforming growth factor
TGF-β	Transforming growth factor beta

TNF-α	Tumor necrosis factor alpha
TNF-β	Tumor necrosis factor beta
VCAM-1	Vascular adhesión molecule-1
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoprotein
VSMC	Vascular smooth muscle cells
ZIA	Zymosan-induced arthritis

Resumen

RESUMEN

La aterosclerosis, una enfermedad cardiovascular inflamatoria crónica, se caracteriza por el engrosamiento de la pared arterial que termina con la formación de una placa de ateroma. En este proceso, el depósito de colesterol, la inflamación, la formación de la matriz extracelular y la trombosis juegan un papel importante. La identificación de las moléculas implicadas en estos procesos podría contribuir al desarrollo de mejoras en los tratamientos para estas patologías. La fosfatasa calcineurina juega un papel clave en la activación de células T, la expresión de citoquinas, el crecimiento y la diferenciación del músculo esquelético y cardíaco, y muchos otros procesos relacionados con la inflamación. Por lo tanto, calcineurina podría representar una diana terapeútica para la prevención de la respuesta inflamatoria que desencadena el proceso aterosclerótico. La hipótesis de este trabajo defiende que la inhibición de la calcineurina en un modelo de ratón de aterosclerosis experimental reduce la progresión de la placa en las primeras etapas de la enfermedad. Se demuestra que la inhibición de la actividad fosfatasa CN, por inhibición farmacológica o mediante la expresión de un péptido inhibidor, reduce la formación de la placa de aterosclerosis en las fases tempranas de esta patología en un modelo animal. Los resultados obtenidos contribuyen a la identificación de la calcineurina como una posible diana terapéutica para la prevención de la progresión de la aterosclerosis.

Este estudio también describe un sistema de expresión lentiviral que contiene el promotor de la E-selectina (ESELp) y permite la expresión del transgen en respuesta a inflamación. La inducción de este sistema lentiviral en situaciones pro-inflamatorias se confirmó en un ensayo de matrigel subcutáneo y en un modelo murino de artritis. Nuestros resultados indican que el sistema lentiviral basado en ESELp, cuya expresión aumenta en los episodios recurrentes de inflamación aguda y disminuye durante la fase de remisión, podría tener una aplicación en el diseño de nuevas estrategias para el tratamiento de enfermedades inflamatorias como la aterosclerosis.

Summary

SUMMARY

Atherosclerosis, a chronic inflammatory cardiovascular disease, is characterized by the thickening of the arterial wall that will end up in the formation of an atherosclerotic plaque. In this process, cholesterol deposition, inflammation, extracellular-matrix formation and thrombosis play important roles. The identification of molecules involved in these processes could aid the development of improved treatments for these pathologies. The phosphatase calcineurin is a key effector of T-cell activation, cytokine gene expression, skeletal and cardiac muscle growth and differentiation, and many other inflammation-related processes. Therefore, it stands as a promising target for preventing the inflammatory response that triggers the atherosclerotic process. We show that the inhibition of calcineurin in a murine model of experimental atherosclerosis reduces plaque progression in early stages of the disease. This inhibition is achieved pharmacologically or by lentiviral delivery of a CN-inhibitory peptide. Our results identify calcineurin as a potential therapeutic target for prevention of atherosclerosis progression.

This study also describes a lentivector containing the E-selectin promoter (ESELp) that selectively drives transgene expression during early inflammation. The specificity of induction of this lentivector under inflammatory conditions was confirmed in a matrigel plug assay and in a murine arthritis model. Our results indicate that the ESELp-based expression system, that is upregulated in acute inflammation episodes and is down-regulated during remission, could have a potential application in the design of new strategies for the treatment of inflammatory diseases such as atherosclerosis.

Introducción

1. ENFERMEDADES CARDIOVASCULARES: EL RETO DEL SIGLO XXI

El término *cardio* proviene del griego καρδιά, kardia, que significa corazón y el término *vascular* procede del latín y significa *conducto* (uās) *pequeño* (-culum). El término *cardiovascular*, por tanto, se refiere a la red de vasos sanguíneos, que realizan una función esencial en el organismo y cuya función se ve afectada en un gran número de patologías descritas. El cáncer, por ejemplo, no puede progresar sin que el tumor forme nuevos vasos, un proceso conocido como angiogénesis. La permeabilidad de los vasos se ve incrementada durante procesos inflamatorios. El daño de los mismos, causado por traumatismo o daño espontáneo, puede derivar en hemorragia debido al daño mecánico producido en el endotelio vascular, que está en contacto con el torrente sanguíneo. Por otro lado, la oclusión de los vasos sanguíneos por la formación de una placa de ateroma, un trombo sanguíneo o un cuerpo extraño, puede conducir a fenómenos isquémicos y necrosis de los tejidos por la falta de oxígeno. Conocemos bien también el significado de enfermedad cardiovascular (CVD-del inglés *cardiovascular disease*), cuáles son los factores de riesgo, qué hábitos saludables nos ayudan a prevenirlas, y qué medicamentos se encuentran a nuestra disposición para su tratamiento.

Con un conocimiento tan amplio sobre las CVD y del sistema cardiovascular es difícil entender cómo pueden ser la primera causa de muerte en el mundo, y que se estime que su prevalencia vaya a seguir aumentando en los próximos años. Un factor muy importante a tener en cuenta es el inmenso gasto económico asociado a la morbilidad y mortalidad de las CVD, que representa actualmente uno de los principales problemas en muchas sociedades por todo el mundo. Se han identificado numerosos factores que contribuyen al desarrollo de las CVD, siendo muchos de ellos una consecuencia de nuestros hábitos. El balance energético ha cambiado en gran parte de las poblaciones en comparación con generaciones anteriores, de forma que el gasto energético de las personas es actualmente mucho menor a las calorías ingeridas. El nuevo estilo de vida que incluye el sedentarismo, el abuso en el consumo de productos hipercalóricos y grasos y el tabaquismo, aumenta el riesgo de obesidad, hipercolesterolemia e hipertensión (Fuster and Vahl, 2010). Dada la gran prevalencia de las CVD, los esfuerzos para evitarlas deberían centrarse en el tratamiento y prevención de los

factores de riesgo implicados en su desarrollo, una estrategia que es al mismo tiempo más fácil y mucho menos costosa que el tratamiento de una CVD en estado avanzado.

2. ATEROSCLEROSIS: UNA CVD INFLAMATORIA CRÓNICA

La aterosclerosis es la CVD más común y la principal causa de muerte en los países desarrollados. Está caracterizada por el engrosamiento y endurecimiento de la pared vascular que afecta a los vasos mayores. Esta enfermedad se ha considerado durante mucho tiempo como un simple depósito de lípidos en la pared arterial, que con el tiempo derivaba en la estenosis (estrechamiento de la luz del vaso) con el consecuente desequilibrio hemodinámico. Sin embargo, hoy en día se sabe por numerosos estudios que la mayoría de infartos de miocardio se originan a partir de la ruptura de placas de ateroma que no se detectaron mediante una angiografía convencional (Fuster and Vahl, 2010).

La aterosclerosis se caracteriza principalmente por un engrosamiento de la pared arterial que deriva en la formación de una placa de ateroma. En este proceso juegan un papel muy importante el depósito de colesterol, la formación de matriz extracellular y los procesos inflamatorios y trombóticos. Es una enfermedad asintomática, hasta etapas muy avanzadas en las que se produce la rotura de la placa y se libera un trombo al torrente sanguíneo causando infarto e isquemia (Libby, 2010).

En los últimos años la inflamación ha cobrado una gran importancia para el mayor entendimiento de esta enfermedad. La aterosclerosis ha dejado de considerarse una enfermedad causada simplemente por depósitos de colesterol que obstruyen las arterias. La finalidad de las nuevas terapias van más allá de diagnosticar una estenosis aórtica y tratarla con técnicas invasivas de revascularización, ya que el mayor peligro de una placa se encuentra en su vulnerabilidad y potencial trombogénico, no simplemente en el grado de estenosis que produce.

2.1 PAPEL DEL ENDOTELIO VASCULAR EN ATEROSCLEROSIS

El endotelio vascular tapiza la cara interna de la pared vascular, conocida como capa Íntima, y se encuentra en contacto con el torrente sanguíneo. El endotelio posee una amplia variedad de propiedades que mantienen activamente la homeostasis vascular, como la hemocompatibilidad, regulación del tono vascular y acciones anti-inflamatorias. Los primeros estudios en aterosclerosis experimental revelan la adhesion de leucocitos a la capa íntima de la arteria como una de las primeras alteraciones morfológicas en el proceso de aterogénesis (Libby et al, 2006).

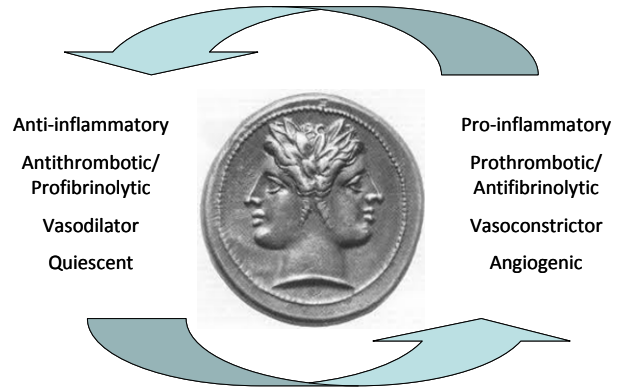

Figura 1. Las dos caras de la célula endotelial. La célula endotelial (EC) se puede representar igual que Jano, el dios romano de los umbrales, de los comienzos y transiciones, dibujada como si tuviera dos cabezas en direcciones opuestas. Estratégicamente situada en la interfaz de la sangre y los tejidos, la EC debe mirar en ambas direcciones. La dualidad entre las propiedades hemostáticas (izquierda) y las funciones que implican la defensa del huésped, lesión de los tejidos y otras enfermedades (derecha) evoca las dos caras de Jano. *Adaptado de Libby et al, 2006a.*

Diversos estudios realizados durante la década de los 80 sobre los mecanismos de adhesión de la célula endotelial (EC) permitieron la identificación de diversas moléculas de adhesión, incluyendo la molécula de adhesión vascular-1 (VCAM-1), la molécula de adhesión intracelular-1 (ICAM-1), y las selectinas P y E; toda ellas se expresan en la superficie de la célula endotelial vascular y median la captura de los leucocitos en sangre. La expresión de VCAM-1 e ICAM-1 aumenta durante la aterosclerosis, especialmente en las primeras etapas de la enfermedad. Las selectinas son moléculas de union a carbohidratos, que se unen a ligandos glicoproteicos y se expresan en ECs, leucocitos y plaquetas. Durante las primeras etapas de la formación de la placa, el endotelio, activado por diversos factores de riesgo, expresa las

selectinas que mediarán la interacción o *rolling* de los leucocitos circulantes con las ECs (Kampoli et al, 2009; Ley, 2003).

La enfermedad vascular es un estado patológico de las grandes y medianas arterias desencadenado por la disfunción endotelial. A causa de factores externos como la presencia de patógenos, la oxidación de lipoproteinas de baja densidad (ox-LDL) y otros estímulos inflamatorios, las ECs se activan provocando una serie de cambios en estas células: comienzan a secretar citoquinas y quimioquinas y a expresar moléculas de adhesion en su superficie, lo que conlleva al reclutamiento de leucocitos (monocitos y linfocitos) que se infiltrarán en la pared vascular. La liberación de citoquinas por las ECs y el reclutamiento de células sanguíneas estimulan la proliferación de las células de músculo liso (VSMCs) y su migración hacia el lumen del vaso. Este proceso contribuye al engrosamiento de la pared vascular y a la formación de una placa constituida por VSMC en proliferación, macrófagos y linfocitos. El engrosamiento de la placa puede provocar una disminución del flujo sanguíneo por el estrechamiento de la luz del vaso, que impedirá el correcto abastecimiento de oxígeno y nutrientes a los órganos. En las fases finales agudas, la rotura de la placa puede conducir a la formación de trombos y provocar un infarto.

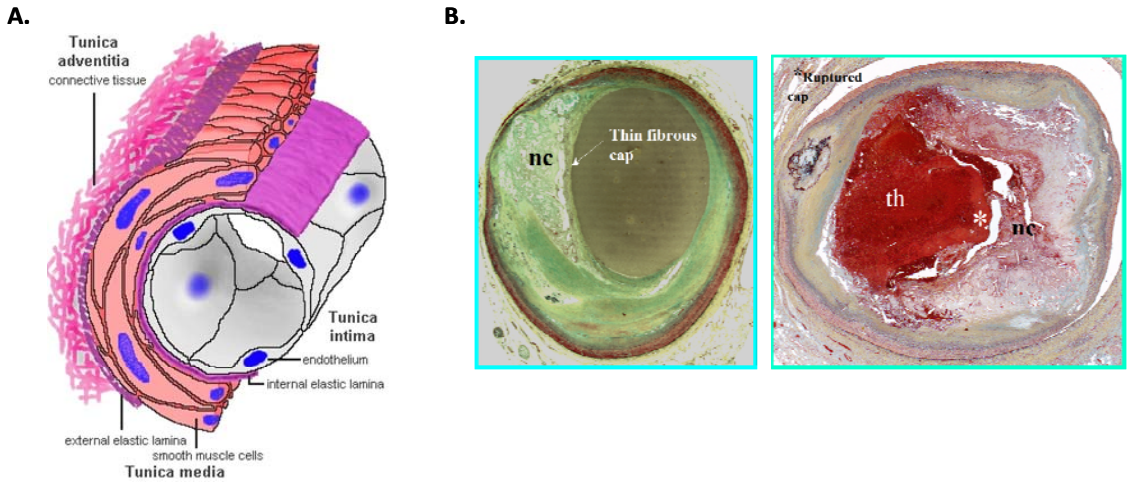

Figura 2. Patología de los vasos sanguíneos. (A) Esquema de la pared de un vaso mostrando sus diferentes capas, desde el endotelio en el interior hasta la adventicia exterior. **(B)** Cortes transversales de un vaso aterosclerótico con una placa vulnerable (izquierda) que se caracteriza por una fina capa fibrosa y un núcleo necrótico (nc), y que termina en la ruptura de la placa (derecha) y la formación de un trombo (th) que ocluye la luz del vaso. *Fotos cortesía del Dr Badimon*.

2.2 INICIO Y DESARROLLO DE LA LESIÓN ATEROSCLERÓTICA

Los procesos inflamatorios dirigen la mayoría de aspectos de la biología de las placas que determinarán su destino clínico. En condiciones de homeostasis, las ECs de la pared vascular son resistentes a la adhesión y agregación de los leucocitos y promueven la fibrinolisis (Libby, 2010). Sin embargo, factores de riesgo como hypertension, hiperlipemia, tabaquismo, hiperglucemia, resistencia a la insulina y obesidad, pueden desencadenar la expresión de moléculas de adhesión que permiten a su vez la adhesión de leucocitos a las ECs de la pared vascular. El fenómeno de *rolling* de los leucocitos por la superficie endotelial constituye el primer paso para su adhesión a las ECs. Diversos estudios han demostrado que este fenómeno de *rolling* está mediado en primer lugar por receptores de adhesión de la familia de las selectinas y sus ligandos (Dong et al, 1998). La selectina L se expresa constitutivamente en la superficie de la mayoría de los leucocitos; la selectina E se encuentra exclusivamente en células endoteliales activadas por citoquinas inflamatorias; la selectina P se libera rápidamente desde gránulos de almacenamiento que se encuentran en plaquetas y ECs. La generación de ratones deficientes en selectinas ha confirmado el papel de las selectinas en el proceso de *rolling* de las células sanguíneas y ha confirmado el solapamiento de funciones de las selectinas durante los procesos inflamatorios (Ley, 2003).

VCAM-1 es la siguiente molécula clave en este proceso. Monocitos y linfocitos interaccionan con las ECs mediante selectinas y VCAM-1, y más adelante migrarán al interior de la placa aterosclerótica en formación (Libby, 2006). La proteina quimio-atrayente de monocitos-1 (MCP-1) es una de las moléculas claves implicadas en la diapedesis o paso de los monocitos a través del endotelio hacia la capa íntima (Hansson, 2005; Hansson and Hermansson, 2011). Los monocitos que penetran la pared arterial expresan receptores *scavenger* para lipoproteínas modificadas, consumen lípidos, y se transforman en células espumosas. El factor estimulador de macrófagos (M-CSF) es un activador de monocitos y un mediador clave en este proceso de transformación. En paralelo, los macrófagos proliferan amplificando así la

respuesta inflamatoria mediante la secreción de numerosos factores de crecimiento y citoquinas, incluyendo el factor de necrosis tumoral (TNF) y la interleuquina 1 (IL-1).

Los linfocitos T también tienen una función clave en el proceso inflamatorio que tiene lugar en el interior de la pared arterial, mediante la producción de citoquinas como interferón-gamma (IFN-γ) ó TNF-α y -β. Este proceso inflamatorio progresa gracias a la activación de leucocitos a través de citoquinas, la liberación de mediadores fibrinogénicos y la proliferación de VSMCs, que influyen en la formación de placas más complejas (Packard and Libby, 2008).

2.3 PROGRESIÓN A LESIONES MÁS COMPLEJAS

Los macrófagos y células T infiltradas en las lesiones ateroscleróticas se localizan en la región protuberante o *shoulder region*, que es la zona de crecimiento de la placa. Así como la acumulación de células espumosas caracteriza las estrías grasas, la acumulación de tejido fibroso define un estado más avanzado de la lesión. Las VSMCs sintetizan la masa de matriz extracellular que caracteriza esta fase en la evolución de la placa (Libby, 2006).

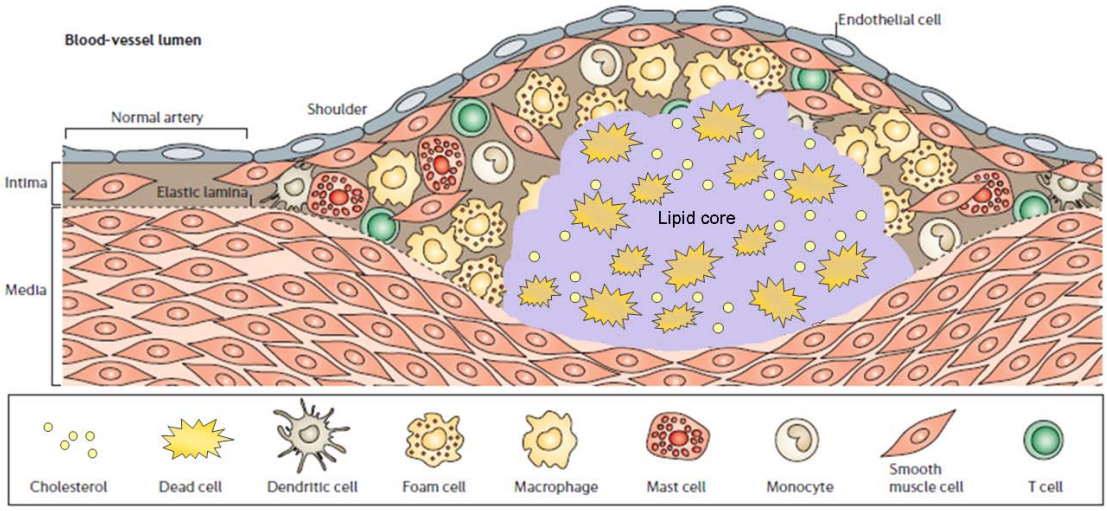

Figura 3. Composición celular de la placa de ateroma. La placa aterosclerótica tiene un núcleo lipídico que contiene colesterol esterificado, cristales de colesterol y restos de células muertas. En torno a él, una capa fibrosa de VSMCs y colágeno ayuda a estabilizar la placa. Las células inmunes como macrófagos, células T y mastocitos localizados en el interior de la placa se encuentran en un estado activado, produciendo citoquinas, proteasas y moléculas pro-trombóticas que contribuyen a la inflamación en la placa y afectan a la función vascular. *Adaptado de Hansonn y Libby, 2006.*

En respuesta a la liberación del factor de crecimiento derivado de plaquetas (PDGF) por macrófagos activados y ECs, y a procesos asintomáticos de semi-rotura de la placa que provocan trombos sin apariencia mural clínica, las VSMCs migran desde la capa media de la pared vascular a la íntima, gracias a la degradación de la matriz extracelular mediada principalmente por la metaloproteasa de matriz 9 (MMP-9). Este proceso de migración de las VSMCs a la íntima causa la evolución de la lesión de placa rica en lípidos a composición fibrótica que puede acabar en calcificación y que será responsable de la estenosis del vaso (Packard and Libby, 2008).

La neovascularización emergente de la red arterial de pequeños vasos (también conocidos como *vasa vasorum*) contribuye también al desarrollo de la lesión de diversas formas. Provee una nueva entrada de leucocitos al interior de la lesión aterosclerótica ya establecida, con el consiguiente riesgo de una hemorragia intraplaca. Esta hemorragia generaría trombina, activando las ECs, monocitos y macrófagos, VSMCs y plaquetas, e intensificando el progreso de formación de placa y favoreciendo complicaciones trombóticas (Libby, 2010).

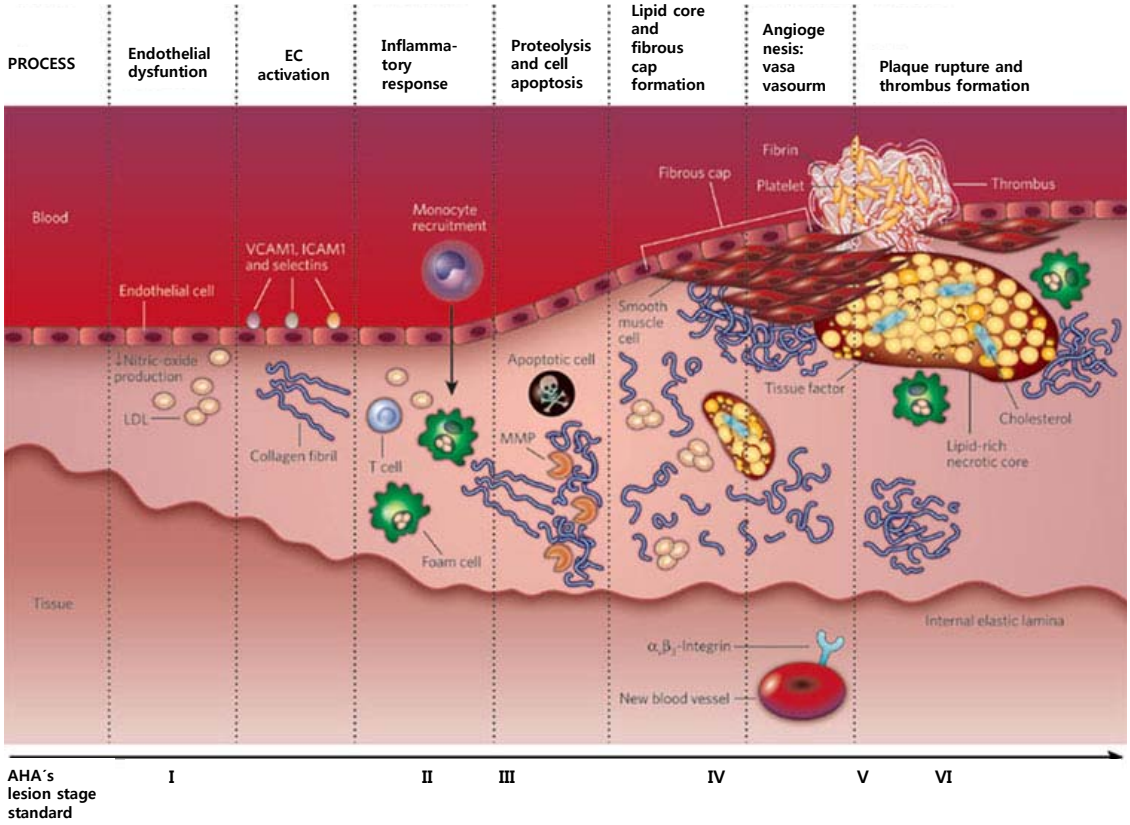

Figura 4. Representación esquemática del desarrollo de la lesión aterosclerótica. Este gráfico muestra un esquema simplificado del desarrollo de la placa de ateroma a partir de un vaso sanguíneo normal (en el extremo izquierdo) que termina en el desarrollo de una placa aterosclerótica compleja y en la formación de trombo luminal (AHA, Asociación Americana del Corazón; ICAM1, molécula de adhesión intercelular 1 ; MMP, metaloproteasas de la matriz; VCAM1, molécula de adhesión celular vascular 1). *Figura adaptada de Sanz y Fayad, 2008.*

2.4 MODELOS ANIMALES PARA EL ESTUDIO DE LA ATEROSCLEROSIS

Numerosas especies animales han sido utilizadas para el estudio de la patogénesis y posibles tratamientos de la aterosclerosis. El primer modelo experimental surgió en 1908 cuando se observó la formación de lesiones ateroscleróticas en aortas de conejos alimentados con una dieta rica en proteínas animales (Jawien et al, 2004). El estudio de esta enfermedad ha estado restringido durante mucho tiempo a animales de gran tamaño, como el cerdo, el conejo o los primates. Los conejos no desarrollan aterosclerosis espontánea pero son muy sensibles a dietas ricas en colesterol y desarrollan como consecuencia lesiones en muy poco tiempo. Estas lesiones, sin embargo, difieren en composición de las humanas ya que tienen un mayor contenido en macrófagos y los niveles de colesterol en plasma son muy elevados. El cerdo

representa un buen modelo debido a su gran similitud al humano tanto en composición de placa como en niveles de colesterol en plasma sanguíneo cuando se les alimenta con una dieta rica en colesterol. A pesar de los grandes avances que se han conseguido en el estudio de esta enfermedad gracias a estos modelos animales, el coste del mantenimiento y gestión de colonias es altísimo. Por este motivo, el uso de un modelo de ratón en el estudio de la aterosclerosis ha supuesto una gran ventaja.

2.4.1 Modelos Murinos para el Estudio de la Aterosclerosis

El modelo de ratón para el estudio de aterosclerosis experimental reúne diversas condiciones que hacen rentable su uso: es fácil de manipular, fácil mantenimiento y ampliación de las colonias, coste muy bajo en comparación con los modelos de animales más grandes y además determinadas cepas desarrollan aterosclerosis en un corto periodo de tiempo. El ratón es un animal muy resistente al desarrollo de aterosclerosis, a excepción de la cepa C57BL/6, que desarrolla lesiones rápidamente cuando se les alimenta con una dieta rica en colesterol. Los primeros estudios realizados en este modelo se llevaron a cabo en los años 60, y fue en esos años cuando Paigen y colaboradores desarrollaron la *dieta Paigen*, rica en grasa y colesterol y con un pequeño porcentaje de ácido cólico. Este modelo fue muy utilizado a pesar de tener una gran desventaja: después de alimentar a los animales con esa dieta durante periodos de 14 semanas a 9 meses, las lesiones que se obtenían eran pequeñas, se localizaban principalmente en la válvula aórtica y diferían de las humanas en su composición. Por ello, la generación de nuevos modelos genéticamente manipulados para modificar y acelerar la progresión de la enfermedad ha supuesto un gran avance.

2.4.1.1 El Modelo de Ratón apoE

Los ratones apoE son deficientes en la apolipoproteína E, una proteína con una función crucial en el metabolismo de las lipoproteínas, debido a la sustitución de parte del gen APOE por un gen resistente a la neomicina (Piedrahita et al, 1992). Esta proteína interviene en el metabolismo y degradación de los quilomicrones procedentes de la dieta y en la eliminación del exceso de lipoproteínas de muy baja densidad procedentes del hígado. Este modelo constituyó el primer modelo experimental de hiperlipemia y aterosclerosis. A pesar de que la aterosclerosis es principalmente una

enfermedad que afecta al ser humano, y dada la gran diferencia que existe entre ambas especies, nunca se pensó que un modelo de ratón podría utilizarse para mimetizar e interpretar esta enfermedad humana. Después de más de una década trabajando con el modelo de ratón, el ratón apoE ha resultado ser altamente fiable para el estudio de placas de ateroma asemejando las humanas, y ha proporcionado una plataforma de estudio en detalle sobre el curso de la enfermedad, así como para la exploración de nuevas terapias (Avani et al, 2009).

El modelo apoE se considera uno de los más relevantes para el estudio de esta enfermedad debido a su elevada hipercolesterolemia y al desarrollo espontáneo de lesiones en arterias, muy similares a las del ser humano. Este proceso se puede acelerar con la administración de una dieta rica en colesterol. Hayek y colaboradores desarrollaron una dieta más fisiológica que la *dieta Paigen* llamada *Western diet* o *dieta occidental* basada en la dieta americana. Con esta dieta se acelera la formación de la lesión y aumenta su tamaño (Figura 5)(Plump and Breslow, 1995;Jaiwen et al, 2004). Las placas de ateroma se forman sistemáticamente en las zonas más vulnerables de los grandes vasos, como la válvula aórtica y el arco aórtico, que son áreas donde el flujo sanguíneo es más turbulento (Crauwels et al, 2003). Gracias a este modelo se han conseguido importantes avances en el estudio de la aterosclerosis como la identificación y modificación de genes susceptibles a esta enfermedad, del papel de determinados tipos celulares con una función importante en el transcurso de la enfermedad, y el diseño de terapias que podrían detener la progresión de la placa e incluso conseguir su regresión (Jawien et al, 2004; Ibañez and Badimon, 2010).

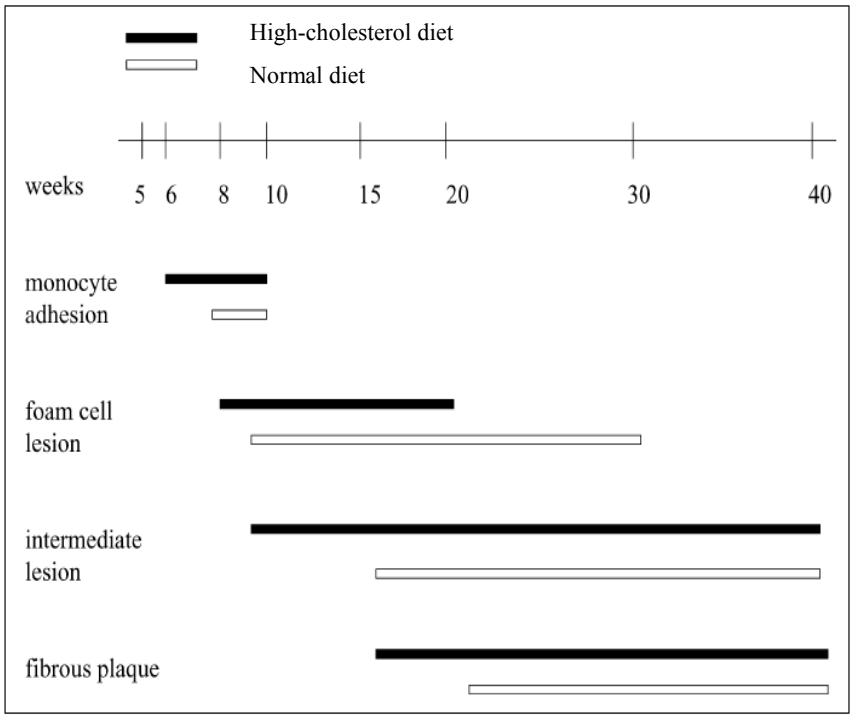

Figura 5. Progresión de la lesión aterosclerótica en el modelo de ratón apoE. Diagrama que muestra cómo la formación de lesiones en ratones alimentados con una dieta alta en colesterol (o dieta occidental) se acelera en comparación con los ratones alimentados con una dieta normal. *Adaptado de Jawien et al, 2004.*

2.4.1.2 Otros Modelos de Aterosclerosis Experimental en Ratón

Los ratones knockout para el receptor de las LDL (LDLR-KO) se generaron para el estudio y tratamiento de la hipercolesterolemia familiar. Estos animales sufren un gran aumento en los niveles de LDL y VLDL con dieta normal sin desarrollar aterosclerosis, pero son muy susceptibles a la *dieta Paigen* que aumenta los niveles de colesterol en plasma y provoca el desarrollo de grandes lesiones en los vasos. Aunque es un modelo menos caracterizado que el modelo apoE, la composición y progresión de la lesión en estos animales es muy parecida a la que desarrollan los ratones apoE, y por tanto, similares a las humanas.

Otro modelo desarrollado recientemente es el doble knockout para APOE y LDLR (APOE/LDLR-DKO); estos animales desarrollan hipercolesterolemia y aterosclerosis más rápido que los animales apoE en dieta normal (Jawien et al, 2004).

En conclusión, los modelos de ratón modificados genéticamente han cambiado radicalmente el enfoque del estudio de la aterosclerosis, permitiendo clasificar la aterosclerosis como una enfermedad inflamatoria crónica.

3. LA FOSFATASA CALCINEURINA

3.1. LA PROTEINA

La proteína calcineurina (CN), purificada a finales de los años 70 por la Dra. Claude Klee, debe su nombre a su regulación por calcio (calci-) y a su abundancia en tejidos neuronales (-neurin) (Klee, 1979). Es una serín-treonín fosfatasa única que se activa por calcio y calmodulina (CaM) y que regula diversas vías de señalización dependientes de calcio en eucariotas, destacando la vía del factor nuclear de células T activadas (NFAT). La CN está implicada en numerosos procesos fisiológicos, como la activación de las células T, la regulación de la expresión de genes de citoquinas, el desarrollo y diferenciación del músculo esquelético y cardiaco, y en procesos de memoria y apoptosis de linfocitos T, ECs, neuronas y macrófagos. Además participa en la regulación de la actividad neurotransmisora en el cerebro, donde constituye el 41% de la proteina total (Aramburu et al, 2000).

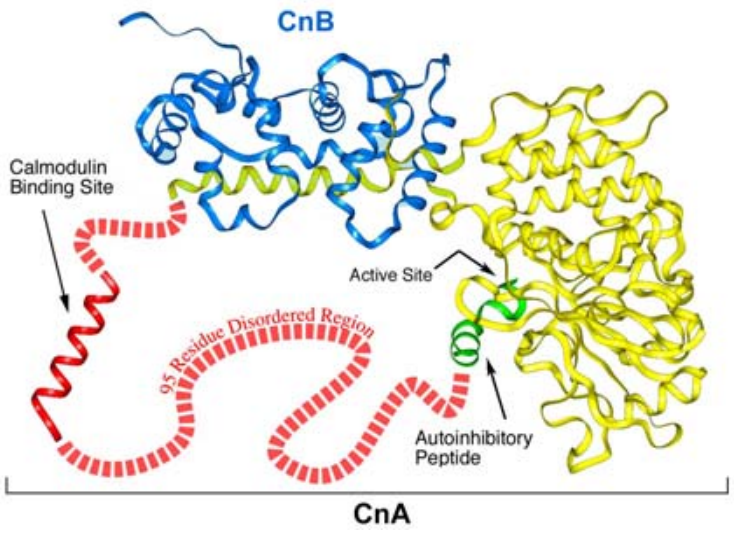

Figura 6. Estructura secundaria de la calcineurina. Representación esquemática de la estructura secundaria del heterodímero CN, que consta de una subunidad reguladora o CnB (en azul) unida no covalentemente a la subunidad catalítica o CnA. La subunidad catalítica contiene el dominio fofatasa y la alfa hélice de unión a CnB (en amarillo). También contiene el dominio autoinhibidor (en verde) que está en contacto con el centro activo. En rojo aparece una región de CnA que carece de estructura secundaria y que une el dominio de unión a CnB con el dominio autoinhibidor. El dominio de unión a calmodulina aparece reflejado como una hipotética alfa hélice formada tras la unión a calmodulina.

CN es un heterodímero que comprende una subunidad catalítica A (CnA) y una subunidad reguladora B (CnB). La CnA contiene el dominio fosfatasa y una región reguladora, ambas conectadas por una secuencia linker de 13 aminoácidos. La región reguladora contiene un dominio de unión a la CnB (BBD), un dominio de unión a la

CaM, y un dominio autoinhibidor (AID) (Klee et al, 1998). La CnB contiene cuatro motivos de unión a calcio (EF-hand) y se une fuertemente a CnA en presencia de concentraciones de calcio sub-micromolares. A concentraciones bajas de calcio la calmodulina (CaM) no está unida al complejo y el dominio AID bloquea estéricamente el centro activo. A medida que aumenta la concentración de calcio, se une a los motivos EF-hand de la CaM, facilitando su interacción con la CnA y produciendo un cambio conformacional de modo que el domino AID se desplaza y deja de bloquear el centro activo (Rusnak and Mertz, 2000).

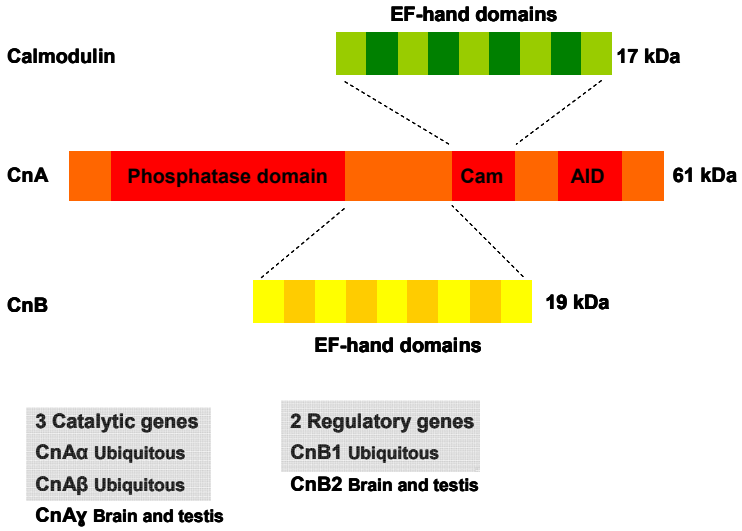

Figura 7. Representación esquemática de las subunidades catalítica (CnA) y reguladora (CnB) de calcineurina y de calmodulina. Esquema de los dominios descritos en las subunidades de CN y de CaM. En vertebrados existen 3 genes que codifican para la subunidad catalítica y 2 genes que codifican para la subunidad reguladora (en gris se destacan las isoformas de CN que contribuyen a la actividad CN en la mayoría de los tejidos). EF-Hand, dominio de unión a calcio; Cam, dominio de unión a calmodulina; AID, dominio autoinhibidor. *Adaptado de Wilkins y Molkentin de 2004.*

3.2 LA RUTA DE SEÑALIZACIÓN CN/NFAT

CN se activa por el aumento de los niveles de calcio en el citosol de la célula, provocando la desfosforilación de varios sustratos, incluyendo NFAT (Crabtree, 1999). La familia de factores de transcripción NFAT consta de cinco miembros descritos, cuatro de los cuales (NFATc1-c4) regulan la transcripción de genes en respuesta a señales de calcio intracelulares que activan CN. Están presentes en muchos tejidos como el endotelio, músculo esquelético y liso, el miocardio y el cerebro. Cuando NFAT no está activo se localiza en el citoplasma celular y está altamente fosforilado. Cuando

CN se activa mediante señales intracelulares de calcio, desfosforila NFAT que, tras exponer su secuencia de localización nuclear, se trasloca al núcleo celular donde activa la transcripción de numerosos genes, incluidas citoquinas y otras moléculas del sistema inmune como la interleuquina-2 (IL-2), IL-3, IL-4, IL-5, TNF-α, factor estimulador de colonias de granulocitos macrófagos (GM-CSF), IL-12 p40, el receptor de IL-2, CD40L, FasL, y CD25 (Rao, 1997). Para controlar la transcripción de estos genes, NFAT se une al DNA e interacciona con otros factores de transcripción (ej. AP-1, MEF-2 or GATA-4) con los que coopera en la regulación transcripcional; estas interacciones a su vez pueden ayudar a estabilizar la localización nuclear de NFAT. La actividad de NFAT en el núcleo finaliza por medio de kinasas intracelulares (ej. p38, JNK, GSK3) que vuelven a fosforilar a NFAT, provocando su transporte de vuelta al citoplasma mediante la vía de exportación nuclear dependiente de la exportina 1, Crm1 (Gomez del Arco, 2000).

La desfosforilación de NFAT requiere de una interacción previa entre NFAT y CN; ambas interaccionan físicamente mediante dos sitios de unión o *docking sites* (Liu et al, 2009; Li et al, 2011). Aramburu y colaboradores descubrieron la existencia de un importante sitio de unión a CN, conservado en los 4 miembros NFATc1-c4, localizado cerca del extremo N-terminal del dominio regulador de NFAT, y que contiene la secuencia consenso ‘‘PxIxIT’’ (‘‘x’’, cualquier aminoácido). Al interferir con el anclaje de CN en la secuencia PxIxIT se inhibe la activación de NFAT y la expresión de genes reporteros dependientes de NFAT (Aramburu et al, 1999). Un péptido de alta afinidad derivado de la secuencia PxIxIT, y conocido como VIVIT, permitió la identificación del sitio de anclaje del motivo PxIxIT en CN; se demostró que VIVIT formaba contactos a lo largo del dominio fosfatasa de la subunidad CnA (Li et al, 2004). El péptido VIVIT era más efectivo que el péptido original inhibiendo la desfosforilación de NFAT mediada por CN *in vitro*, sin interrumpir otras vías de señalización dependientes de CN (Aramburu et al, 1999).

Aparte del motivo PxIxIT, se identificó un segundo motivo de unión a CN que se localiza cerca del extremo carboxilo de la región reguladora de NFAT (Liu et al, 1999; Liu et al, 2001; Park et al, 2000; Martinez-Martinez et al, 2006). Este motivo contiene la

secuencia consenso LxVP y, junto con PxIxIT, contribuye a la afinidad global del sustrato por CN; variaciones en la secuencia LxVP modificaran la unión de NFAT a CN (Martinez-Martinez et al, 2006). A diferencia del motivo PxIxIT, LxVP inhibe la actividad fosfatasa de CN y require la unión de la subunidad A (CnA) con la B (CnB) para su interacción.

Se ha demostrado que el péptido LxVP se une a un bolsillo hidrofóbico en la interfase de las 2 subunidades de CN (Rodriguez et al, 2009). Esta superficie hidrofóbica de interacción con CN formada en la región de unión de CnA con CnB es también esencial para la unión de los fármacos inmunosupresores ciclosporina A (CsA) y FK506, administradas frecuentemente después de un transplante (Griffith et al, 1995; Huai et al, 2002; Jin and Harrison, 2002; Kissinger et al, 1995). A diferencia de CsA y FK506 que necesitan formar complejos con inmunofilinas celulares específicas para poder unirse a CN, el péptido LxVP interacciona directamente con la superficie de anclaje a CN descrita anteriormente (Rodriguez et al, 2009). Por ello, se podría esperar de este péptido que afecte exclusivamente a la actividad fosfatasa de CN, no teniendo efecto sobre otras rutas no dependientes de CN que sí se ven afectadas por el tratamiento con CsA o FK506. Por tanto, cabría esperar que el tratamiento con el péptido LxVP tuviese menos efectos secundarios que los tratamientos con CsA o FK506. Además, este péptido podría ser una herramienta muy útil para identificar los efectos secundarios de CsA y FK506 que no estén relacionados con la inhibición de la actividad fosfatasa de CN.

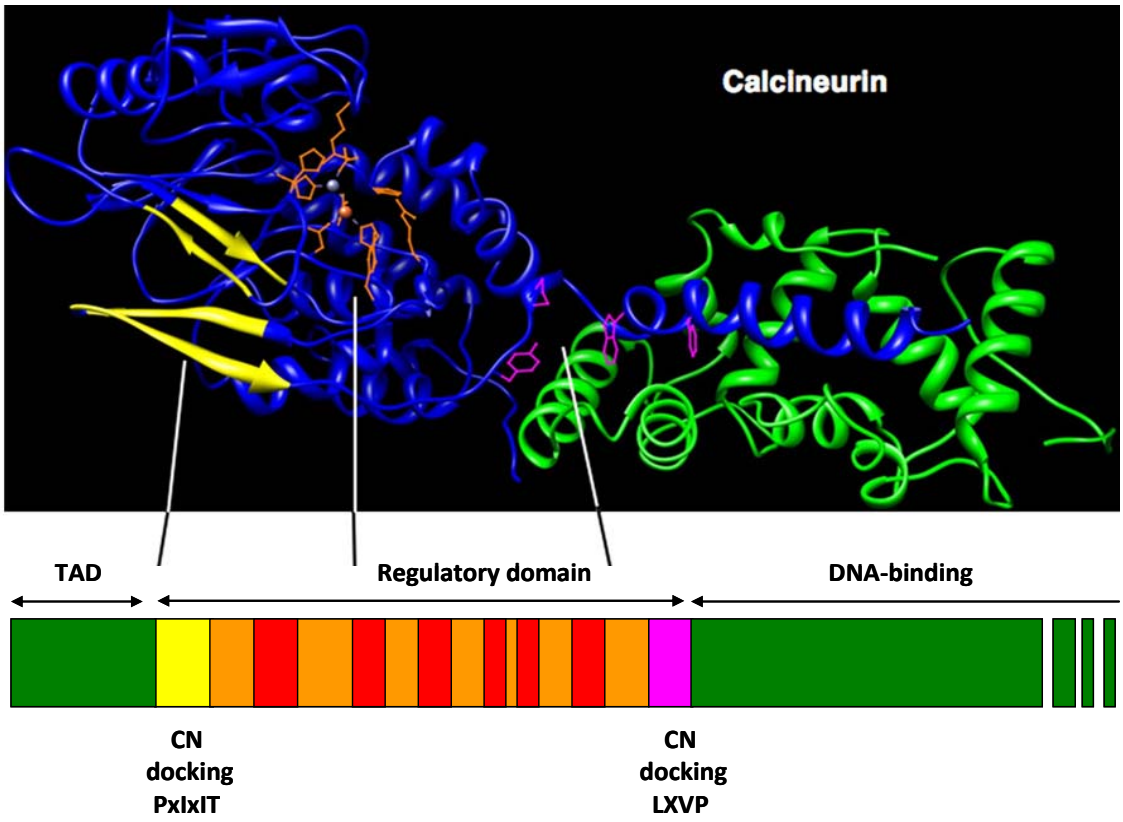

Figura 8. Los sitios de interacción entre CN y NFAT mostrados mediante la estructura cristalográfica de CN y la representación esquemática de NFAT. (Arriba) Estructura secundaria de CN. En azul, subunidad catalítica o CnA; en verde, subunidad reguladora o CnB; en amarillo, sitio de unión del motivo PxIxIT en CnA; en naranja, centro activo del dominio fosfatasa de CnA; en rosa, superficie de unión del motivo LxVP sobre la superficie compuesta de las dos subunidades de la calcineurina. (Abajo) Esquema de NFAT donde se indican los diferentes dominios: Dominio Regulador que contiene la secuencia PxIxIT (amarillo), la secuencia LxVP (rosa) que flanquean los múltiples sitios de fosforilación (rojo) del dominio regulador de NFAT. El dominio de transactivación (TAD) se localiza en el extremo N-terminal y en el extremo C-terminal se encuentra el dominio de unión a ADN (DNA-binding), ambos indicados en color verde. *Adaptado de Liu et al, 2009; Li et al, 2011.*

3.3 LA RUTA CALCINEURINA/NFAT Y LAS ENFERMEDADES CARDIOVASCULARES

Hast la fecha se han descrito cinco miembros de NFAT (NFATc1 a NFATc5), de los cuales, 4 de ellos (NFATc1-c4) se expresan en el sistema cardiovascular. Los miembros NFATc3 y NFATc4 son activos durante procesos patológicos que afectan al sistema cardiovascular, incluyendo fibrilación auricular e hipertrofia. Numerosos estudios han identificado alteraciones en los niveles de calcio en insuficiencias cardiacas de forma que la señal intracelular de calcio disminuía y se prolongaba. Estos estudios plantean la hipótesis de que alteraciones en los niveles de calcio intracelular pueden provocar progresivamente una hipertrofia o cardiomiopatía, en parte debido a una activación constante de rutas de transducción dependientes de calcio. La reciente identificación

de la CN como un factor regulador de hipertrofia confirma esta hipótesis (Molkentin, 2000).

A pesar de haber estudiado intensivamente el papel de la ruta CN/NFAT en el desarrollo de hipertrofia cardiaca, se sabe mucho menos sobre su función en vasos sanguíneos o su posible implicación en aterosclerosis u otras enfermedades cardiovasculares (Wilkins et al, 2004). En el sistema vascular, los miembros de NFAT (c1 y c3) están implicados en el crecimiento celular, el remodelamiento de VSMCs, el control del desarrollo vascular y la angiogénesis; NFAT también se activa en respuesta a procesos inflamatorios y a una presión intravascular elevada. En el endotelio, NFAT controla la expresión génica durante el remodelamiento y se activa por factores de crecimiento (Hernandez et al, 2001; Boss et al, 1998). Sin embargo, las señales de calcio precisas, fisiológicas o patológicas, que causan la activación de la ruta CN/NFAT en células endoteliales todavía no se han determinado (Rinne et al, 2009). El papel crítico que juega CN en la regulación de células T y la expresión génica de citoquinas, así como sus implicaciones en rutas dependientes de calcio, nos conduce a la hipótesis de implicar a CN en el desarrollo de CVDs como la aterosclerosis o la restenosis.

4. LA CALCINEURINA COMO DIANA TERAPEÚTICA

CN actúa como un regulador multifuncional de distintas dianas como NFAT, NF-κB, factor activador de monocitos-2 (MEF2), y el factor apoptótico Bad. NFAT se identificó inicialmente en linfocitos T como un regulador esencial de la expresión de IL-2 (Shaw et al, 1988). Más adelante, también se implicó a NFAT en la diferenciación de osteoclastos, en la especialización del tipo de fibra muscular, y en el desarrollo de las válvulas cardiacas y miocardio. CN activa NFAT no solo en células T, sino también en otras células del sistema inmune como las células B o las células citotóxicas naturales (*natural killer*); también regula a NFAT en la mayoría de células vasculares como VSMCs, ECs y macrófagos. Estas funciones destacan a NFAT como un importante modulador del desarrollo y de la función del sistema cardiovascular (Yu, 2007).

La ruta de señalización CN/NFAT ha surgido como una diana terapeútica crucial en la regulación de inflamación e hiperplasia del miocardio y de las VSMCs. La inhibición selectiva de NFAT se considera beneficiosa tanto en cardiopatías como en

vasculopatías (Yu, 2007). Dada la importante función de la ruta de CN en distintos procesos fisiológicos e inmunológicos, su inhibición se considera una modalidad de terapia muy efectiva en el tratamiento de transplantes para evitar rechazo, en enfermedades autoinmunes y en alteraciones cardiovasculares.

4.1 INHIBICIÓN FARMACOLÓGICA DE LA CALCINEURINA

CN es diana de los inmunosupresores CsA y FK506 (ó Tacrolimus), que actúan impidiendo el rechazo tras el transplante de órganos y se utilizan en tratamientos fundamentales para las recuperaciones post-transplante (Liu, 1991). Como herramientas de investigación, CsA y FK506 han permitido la identificación de distintas funciones de CN en diversos procesos biológicos, incluyendo respuestas a estrés celular y tolerancia a medicamentos en hongos; en mamíferos CN está implicada en la respuesta inmune, en la función de células beta pancreáticas, en el desarrollo temprano vascular, en funciones neurológicas y en la diferenciación muscular. Además de su aplicación en la prevención del rechazo post-transplante, los inhibidores de CN se utilizan con éxito en el tratamiento de dermatitis atópica, asma severo, glomeruloesclerosis segmental y artritis reumatoide (Reynolds, 2002; Powell, 2001; Cattran, 2007; Suzuki, 2010).

CsA y FK506 se unen a CN e inhiben su actividad fosfatasa. La unión a CN de estos dos fármacos se realiza mediante la formación de complejos con inmunofilinas específicas: ciclofilina (Cyp) en el caso de CsA y la proteina de unión a FK506 (FKBP) en el caso de FK506. Las inmunofilinas (IPs) se unen a CN exclusivamente en presencia de las moléculas inmunosupresoras (IS); son prolil-isomerasas y su actividad parece no estar relacionada con la inmunosupresión. Diversos estudios estructurales, bioquímicos y genéticos han demostrado que los complejos inmunosupresor-inmunofilina (IS-IP) interactúan con una superficie hidrofóbica formada en la unión de las dos subunidades de CN. Un grupo de aminoácidos presentes en CN se encargan del reconocimiento de estos dos complejos IS-IP (Ke and Huai, 2003). Siendo la CsA (un péptido cíclico) y el FK506 (un macrólido) estructuralmente distintos, su interacción con un dominio común en CN indica que esta región podría ser una región de reconocimiento para otras proteínas de unión a CN (Rodriguez et al, 2009).

4.1.1 CICLOSPORINA A (CsA)

CsA es un undecapéptido cíclico fúngico descubierto en los años 70 y que se utiliza frecuentemente en pacientes transplantados o con alteraciones autoinmunes (Wenger & Payne, 1989). Originariamente la CsA se aisló de cultivos del hongo *Tolypocladium inflatum,* crecido a partir de una muestra de tierra recogida en el sur de Noruega. Rápidamente se demostró que la CsA era muy efectiva en la mayoría de modelos animales de transplantes y enfermedades autoinmunes. La introducción de CsA en 1983 para el tratamiento de un transplante renal alogénico tuvo un gran impacto en el campo de los transplantes de órganos.

El tratamiento con CsA conlleva una serie de efectos secundarios, como son la neurotoxicidad, diabetes e hipertensión (Robert et al., 2010). La mayor toxicidad observada en modelos animales es la nefrotoxicidad, que se manifiesta con la disminución de la velocidad de filtración glomerular, asociada con una reducción del flujo de sangre renal y refleja un aumento en la resistencia vascular. La vasoconstricción producida por la CsA no se limita únicamente al hígado, sino que también afecta a la circulación sistémica.

Una característica a destacar de la CsA es que también se ha asociado con una elevada susceptibilidad al desarrollo de aterosclerosis e hiperlipemia. Muchas de las células y rutas intracelulares implicadas en aterosclerosis pueden verse afectadas por el tratamiento con CsA. Los estudios demuestran que la CsA afecta a múltiples moléculas de señalización que a su vez afectan al metabolismo de lípidos, a rutas de inflamación y secreción en VSMCs, macrófagos, ECs y células T. Aunque se esperaría que la inmunosupresión por parte de CsA tuviese un efecto anti-aterogénico, la diversidad de las rutas afectadas impide llegar a conclusiones definitivas (Kockx et al, 2010).
A pesar de su gran efectividad como inmunosupresor, CsA afecta también muchas rutas celulares que no están asociadas a la inmunosupresión, muchas de las cuales pueden asociarse a los efectos secundarios cardiovasculares que se observan en pacientes transplantados. El tratamiento con CsA causa efectos muy complejos en el metabolismo de lipoproteinas y la producción de ácido biliar; también afecta a las ECs, VSMCs y macrófagos, siendo todos críticos en el proceso de aterosclerosis. En las ECs

inhibe la actividad transcripcional de NFAT, bloquea la inducción de la vía del factor de crecimiento de endotelio vascular A (VEGF-A), inhibiendo su proliferación y migración.

Diversos estudios, como los desarrollados por Satonaka y colaboradores (2004) y el grupo de Liu (2005), han demostrado que CsA inhibe la proliferación y migración de VSMCs tanto *in vitro* como *in vivo,* sugiriendo que podría tener un efecto anti-aterogénico (Kockx et al, 2010). Además, cabe destacar que la expresión de MCP-1 inducida por angiotensina ll en VSMCs se inhibe en presencia de CsA, y por tanto impide la infiltración de macrófagos, deteniendo así la formación de la placa (Satonaka et al, 2004). CsA afecta a diversas vías inflamatorias relacionadas con los macrófagos; inhibe a los macrófagos y monocitos potencialmente activos que desencadenarían reacciones autólogas y alogénicas de linfocitos T, así como la secreción de apoE por parte de las células espumosas en humanos (Kockx et al, 2010).

4.1.2.FK506

La búsqueda de inmunosupresores más seguros y efectivos llevó al descubrimiento de FK506 en un medio de fermentación de *Streptomyces tsukubaensis*, un organismo obtenido a partir de muestras de tierra recogidas en la zona de Tsukuba, al norte de Japón. En general, FK506 ha resultado ser más potente que CsA en una variedad de modelos experimentales de transplante y autoinmunidad; pero, a excepción de algunas diferencias experimentales menores, ambos compuestos tienen un espectro de actividad similar en dichos modelos. Aunque en estudios realizados en distintos laboratorios indican que este nuevo compuesto produce cambios de comportamiento, nefrotoxicidad y toxicidad gastrointestinal severa en ratas y daño del miocardio en perros, en primates se han descrito escasas patologías. Con este historial de toxicidad, los ensayos clínicos con FK506 comenzaron centrándose primeramente en los transplantes de hígado (Sigal and Dumont, 1992).

Los principales atributos de FK506 incluyen su potencia, su capacidad para retrasar o detener la progresión en las primeras fases de rechazo crónico, y su eficacia en el tratamiento y la prevención de los episodios de rechazo agudo, sin recurrir a terapias inmunosupresoras auxiliares. El uso de FK506 permite una disminución más rápida de

esteroides que la terapia con CsA, lo que sugiere la posibilidad de su aplicación como monoterapia en otras condiciones clínicas (Sigal and Dumont, 1992).

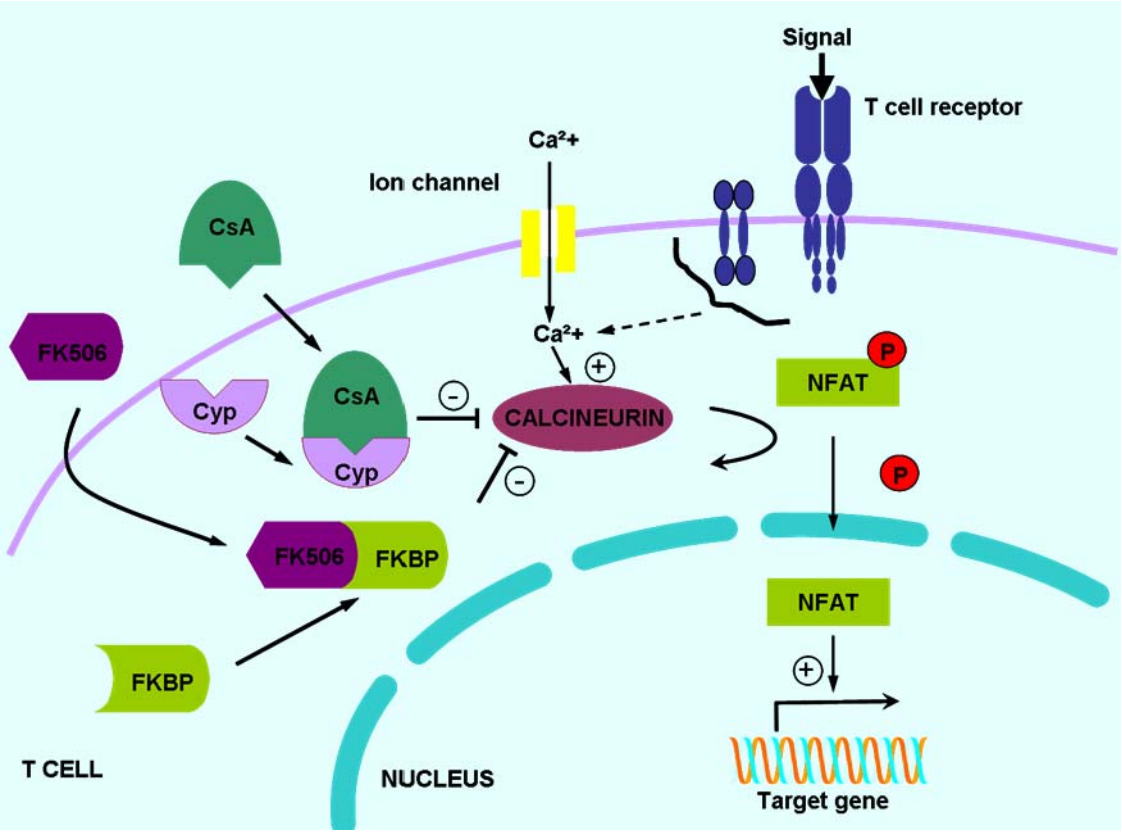

Figura 9. Mecanismo de acción de la CsA y el FK506. A pesar de que CsA y FK506 tienen estructuras químicas relacionadas, sus mecanismos de acción, efectos biológicos y sus efectos clínicos secundarios son muy similares. Dada su naturaleza hidrofóbica, estos compuestos se difunden a través de la membrana plasmática, y una vez en el citoplasma, CsA y FK506, se unen a pequeñas proteínas llamadas ciclofilina A (CyP) y FKBP, respectivamente. Los complejos CsA-CyP y FK506-FKBP, se asocian a CN e inhiben su actividad fosfatasa. Como consecuencia, se bloquea la translocación de NFAT al núcleo, impidiendo la regulación transcripcional de sus genes diana. *Adaptado de Sigal y Dumont, 1992.*

4.1.1.1 Papel de los Inmunosupresores en la Aterosclerosis

El vínculo entre la progresión aterogénica y CN proviene de la aceleración de la aterosclerosis observada en los pacientes receptores de trasplante renal. Las enfermedades cardiovasculares son responsables de aproximadamente el 40% de las muertes en pacientes con transplante renal. Además, existe una correlación bien establecida entre los niveles de lípidos y los eventos cardiovasculares en poblaciones no-transplantadas (Ramezani et al, 2007). El tratamiento con las dosis recomendadas de CsA se asocia con nefrotoxicidad, dando lugar a una disfunción renal a largo plazo, hipertensión e hiperlipidemia (Ekberg et al, 2007). El papel exacto de la CsA en la causa

de la hiperlipidemia después del trasplante de órganos no está claro ya que estos pacientes tienen múltiples factores que contribuyen a ella, como la obesidad post-trasplante y el uso recurrente de corticosteroides (Fernandez-Miranda et al, 1998). Estudios aleatorios con administración de CsA o FK506 después de un trasplante renal o hepático han demostrado hiperlipidemia en pacientes tratados con cualquiera de los agentes, siendo en general más severos en el caso de la CsA (Ichimaru et al, 2001).

CsA y FK506 inhiben la actividad de la fosfatasa CN y su administración a largo plazo se asocia con un mayor riesgo de aterosclerosis (Apanay, 1994; Sutherland, 1995; Ghanem, 1996; Cofán, 2002; Venkiteswaran, 2001). Sin embargo, el hecho de que el uso de estos medicamentos inhiba o atenúe el desarrollo de la aterosclerosis causada por transplante sigue siendo confuso, ya que los resultados obtenidos en diferentes estudios que abordan el efecto de CsA y FK506 en aterosclerosis *de novo* en modelos animales son contradictorios (Drew, 1995; Matsumoto, 1998 ; Roselaar, 1995). Estas diferencias pueden deberse a aspectos singulares de cada modelo, pero también a los efectos inherentes e independientes de CN asociados tanto a CsA como a FK506. Ambos medicamentos se unen a las IPs y pueden alterar distintos procesos biológicos, tales como la permeabilidad mitocondrial o la función de los receptores de rianodina que participan en la homeostasis del calcio intracelular. Un estudio reciente muestra que dosis bajas de FK506 inhiben la progresión de la aterosclerosis inducida por estrechamiento de la carótida (*collar-induced atherosclerosis*) y estabilizan las placas en los ratones deficientes en apoE (Donner, 2005). Estos datos sugieren que FK506 tiene un efecto directo sobre la composición de la placa y no a través de un efecto inmunosupresor sistémico. Dado que FK506 no sólo comparte la eficacia clínica con CsA, sino también muchos efectos secundarios, incluyendo alteraciones en el metabolismo de los lípidos, el uso prolongado de estos fármacos en muchas enfermedades crónicas, a excepción del transplante de órganos o dermatitis atópica, ha sido restringido.

4.2 NUEVOS INHIBIDORES DE CALCINEURINA: ¿SON LA ALTERNATIVA A LOS INMUNOSUPRESORES ACTUALES?

El papel central de CN y de la ruta CN/NFAT en la respuesta inmune sugiere que la interrupción de esta vía de señalización podría representar una alternativa terapéutica a los actuales fármacos inmunosupresores (Kang et al, 2005). Como ya se ha mencionado en este trabajo, CsA y FK506 interrumpen la actividad fosfatasa de la CN afectando a toda la cascada de vías de transducción dependientes de ella. Esta inhibición no selectiva podría dar lugar a efectos secundarios no deseados y toxicidad, tales como la pérdida progresiva de la función renal, hipertensión y neurotoxicidad. Todavía no está claro hasta qué punto estas toxicidades se deben a la inhibición de NFAT, a la interferencia con la desfosforilación de otros sustratos de CN, o a vías potencialmente independientes de CN, como la regulación de TGF-β (Aramburu, 1999).

Por ello, el desarrollo de inhibidores selectivos (por ejemplo, pequeñas moléculas orgánicas y péptidos) que inhiban la unión de CN y NFAT sin afectar a su actividad catalítica sería ideal para el manejo adecuado de las reacciones mediadas por NFAT en diferentes enfermedades (Im and Rao, 2004). Inhibidores de NFAT con menos toxicidad que CsA y FK506 podrían ser útiles para el tratamiento de enfermedades crónicas como la hipertrofia cardiaca, alergias, artritis y enfermedades autoinmunes.

4.2.1 Moléculas Orgánicas de Pequeño Tamaño

Las aplicaciones de moléculas que unen proteínas con alta afinidad se están estudiando intensamente debido a sus innumerables aplicaciones de investigación bioanalítica en biotecnología y biomedicina. Las moléculas orgánicas de pequeño tamaño se preparan mediante síntesis orgánica y su peso molecular es menor que el de las proteínas. Son muy eficientes como inhibidores de las interacciones intracelulares proteína-proteína permitiendo su manipulación química para conseguir una estrecha unión a la superficie de la proteína diana. Además, los inhibidores efectivos se pueden adaptar químicamente para mejorar la estabilidad, eliminar efectos secundarios inespecíficos, y mejorar su distribución *in vivo* (Roehrl et al, 2004).
Se han descrito varios inhibidores de la asociación NFAT-CN (compuestos INCA) que impiden la unión de NFAT o del péptido VIVIT a CN (Kang et al, 2005). Aunque su sitio

de unión se encuentra en el dominio fosfatasa de la CnA, éste no coincide con el sitio central de unión de PxIxIT. Por lo tanto, la inhibición que ejercen estos compuestos ha de ser por un cambio alostérico que tiene un efecto sobre el sitio de unión a PxIxIT. A pesar de los recientes avances en esta tecnología, las pequeñas moléculas orgánicas han tenido escaso impacto en el campo de diagnósticos ya que la mayoría de los ensayos se han interrrumpido en las últimas etapas debido a una baja eficacia y a una toxicidad inesperada.

4.2.2 Péptidos Inhibidores de la Calcineurina

Numerosas evidencias sugieren que el *targeting* de la interacción proteína-proteína CN-NFAT produciría una inhibición selectiva. Varios grupos de investigación han demostrado que la interacción CN-NFAT se basa en el reconocimiento de las secuencias PxIxIT y LxVP de NFAT y que estos reconocimientos son esenciales para una señalización eficiente. La reciente descripción y mejora de péptidos inhibidores selectivos para CN abre nuevas perspectivas para el diseño de nuevos tratamientos de trastornos cardiovasculares.

4.2.2.1 VIVIT

Aparte del *targeting* del sitio catalítico de CN, como la CsA y FK506, una estrategia alternativa ha sido la inhibición selectiva de la activación de NFAT a través del bloqueo de su interacción con CN a través del motivo PxIxIT; NFAT debe unirse a CN a través de los sitios de interacción descritos, LxVP y PxIxIT, antes de ser desfosforilado y translocado al núcleo. El bloqueo específico de la unión entre CN y NFAT permitiría una inactivación eficiente de NFAT, sin afectar a la actividad fosfatasa de CN de forma general (Yu et al, 2006).

La optimización del motivo PxIxIT través de la selección por afinidad de una péptidoteca llevó al descubrimiento del péptido VIVIT, que inhibe la interacción CN-NFAT de forma selectiva y efectiva (Aramburu et al, 1999). Hasta ahora, no se conocen efectos secundarios tóxicos producidos por VIVIT. De hecho, debido a su alta especificidad para interferir con la señalización NFAT y sin afectar a la actividad fosfatasa de la CN, se espera que VIVIT sea mucho menos tóxico que la CsA o FK506 (Yu et al, 2007). Acorde con la información descrita en la tabla que se muestra a

continuación, VIVIT presenta un perfil mucho menos tóxico que los inmunosupresores actuales, CsA y FK506.

Charasteristic	VIVIT	CsA	FK506
Composition	16 member linear L-peptide	Cyclic non-ribosomal undecapeptide	23-member macrolide lactone
M.W.(g/mol)	1684	1203	804
Calcineurin-binding site	Calcineurin docking site on N-terminal regulatory domain of NFAT	Calcineurin active site	Calcineurin active site
Calcineurin phosphatase	Intact	Inhibited	Inhibited
Binding partner	None	Cyclophilin	FK506-binding protein (FKBP-12)
Side effects	Not observed	Nephrotoxicity, hypertension, neurotoxicity	Nephrotoxicity
Administration	Intraperitoneal, subcutaneous	Oral, intravenous (i.v.)	Topical, oral, i.v.
Potential application	Allograft transplantation, cardiac hypertrophy, restenosis	Transplantation, psoriasis, rheumatoid arthritis	Transplantation, eczema

Tabla 1. Características físicoquímicas de VIVIT, CsA y FK506. Con unas aplicaciones clínicas similares a CsA y FK506, VIVIT no muestra efectos secundarios ni necesita formar compuestos para su unión a CN. *Adaptado de Yu et al, 2007.*

Como se ha mencionado anteriormente, NFAT desempeña un papel crítico en la regulación de la transcripción de genes mediada por células T durante la respuesta inmune e inflamatoria. Su activación induce la expresión de muchas citoquinas, las cuales contribuyen al desarrollo de CVDs como la aterosclerosis (Rao et al, 1997). Actualmente, sigue sin estar claro si la inhibición de la señalización mediada por CN-NFAT ayudará a prevenir o a mejorar la aterosclerosis inducida post-transplante. La inhibición selectiva de NFAT por el péptido VIVIT parece ser una estrategia eficaz para disminuir la inflamación y la hipertrofia de forma coordinada. En comparación con CsA y FK506, VIVIT muestra un perfil terapéutico favorable, ya que no afecta a la señal extracelular mediada por PKC ni a la actividad de NF-kB mediada por CN; y su efectividad en la interrupción de la señalización mediada por NFAT es igual. No sólo presenta menor toxicidad que CsA o FK506, VIVIT también constituye una herramienta útil en la exploración de la ruta de señalización CN/NFAT, y podría abrir las puertas al

uso de nuevas terapias para el tratamiento de trastornos cardiovasculares como hipertrofia cardiaca, restenosis y aterosclerosis (Yu et al, 2007).

4.2.2.2 LxVP

Rodríguez y colaboradores (2009) demostraron que un péptido basado en la secuencia LxVP de NFAT inhibe tanto la actividad de la fosfatasa CN hacia un fosfopéptido *in vitro*, y la activación de NFAT y por tanto la transcripción NFAT-dependiente en cultivos celulares. Sorprendentemente, el mismo péptido LxVP estimula, en lugar de inhibir, la actividad fosfatasa de CN hacia una pequeña molécula, el para-nitrofenilfosfato (pNPP); este efecto paradójico es una reminiscencia de lo que ocurre con los complejos IS-IP (Liu et al, 1991). De hecho, el péptido LxVP puede competir con los dos complejos IS-IP por la unión a CN, lo que sugiere que el motivo LxVP podría interactuar con la misma superficie hidrofóbica, formada entre las dos subunidades de CN. Esta hipótesis fue confirmada mediante el análisis de la unión de proteínas de CN mutadas en esta superficie hidrofóbica; los mutantes de CN que mostraron una menor interacción con complejos IS-IP también afectaban a su unión con el péptido LxVP (Rodriguez et al, 2009). Dado que la superficie hidrofóbica de acoplamiento de la CN es también esencial para la unión de los complejos IS-IP (CsA y FK506), los autores proponen que estos medicamentos bloquean la interacción de CN con motivos del tipo LxVP comunes a sustratos de CN, por lo que inhiben la señalización CN-dependiente.

Ya se ha mencionado anteriormente en este trabajo la contribución de LxVP y PxIxIT a la afinidad general del sustrato por CN. Las propiedades de LxVP, sin embargo, son distintas a las de PxIxIT. Los péptidos que contienen cualquiera de estos dos motivos inhiben con eficacia la desfosforilación de NFAT; sin embargo, un péptido que contiene la región LxVP de NFAT impide específicamente no sólo la interacción CN-NFAT sino también la actividad de CN. Las mutaciones en la región de unión de LxVP en la CN de levaduras impiden su función y su interacción con sustratos. Entre los sustratos afectados por estas mutaciones se encuentra Rcn1, presente en levaduras y perteneciente a la familia RCAN; esta familia de proteínas reguladoras de CN está muy conservada y contiene una región de consenso LxVP necesaria para la unión a CN. Se

han identificado secuencias LxVP en distintos sustratos de CN en mamíferos, como RCAN o la kinasa supresora de Ras 2 (KSR2) (Roy and Cyert, 2009).

5. LA TERAPIA GÉNICA COMO TRATAMIENTO DE ENFERMEDADES CARDIOVASCULARES

A pesar de los notables avances terapéuticos de los últimos 30 años, las CVDs siguen siendo la principal causa de discapacidad y muerte prematura. Nuevas herramientas en desarrollo para modificar genéticamente el sistema cardiovascular ofrecerán nuevas posibilidades para el tratamiento de estas enfermedades. La transferencia de genes o terapia génica se puede utilizar como una estrategia para silenciar o sobre-expresar genes involucrados en la progresión de la enfermedad. El potencial terapéutico de esta nueva herramienta se ha demostrado en varios modelos animales de enfermedades cardiovasculares. A pesar de estos avances, todavía hay que mejorar la ingeniería de los vectores para su aplicabilidad en el tratamiento clínico de enfermedades cardiovasculares (revisado en Melo, 2005).

Los problemas actuales de la terapia génica incluyen la falta de especificidad de tejido y la expresión transitoria del gen expresado o transgen, haciéndolos poco adecuados para su aplicación en enfermedades cardiovasculares crónicas. Los vectores no virales presentan bajo rendimiento y baja eficiencia de transferencia génica, ya que no son integrativos y se degradan rápidamente. Los vectores más adecuados para la transferencia de genes en los tejidos cardíacos son los derivados de virus recombinantes. Con estos vectores se generan partículas virales no replicativas que distribuyen los transgenes con alta eficiencia. Entre estos vectores virales, los derivados de virus adeno-asociados (AAV) o de lentivirus destacan por su capacidad de mantener la expresión del gen terapéutico, por lo que los hace aptos para su aplicación el tratamiento de enfermedades crónicas.

La principal preocupación actual se centra en el desarrollo de vectores específicos de tejido que dirijan el transgén terapéutico a un tejido diana y que su expresión responda a los estímulos fisio-patológicos, tales como hipoxia ó estrés oxidativo. Se están desarrollando nuevas estrategias para la distribución local de genes vasculares

mediante *stents* (catéteres) recubiertos de células creadas mediante ingeniería genética, plásmidos o vectores virales que expresan genes terapéuticos (Melo, 2005).

Considerando la aterosclerosis como la enfermedad cardiovascular más común y sabiendo que la inflamación está presente en todas las etapas de la aterosclerosis, los sistemas de vectores regulados por inflamación constituyen una herramienta prometedora para el desarrollo de nuevas estrategias de transferencia génica para las enfermedades cardiovasculares.

5.1 SISTEMAS REGULADOS POR INFLAMACIÓN

Las enfermedades inflamatorias crónicas se caracterizan por episodios de recurrencia y remisión que a menudo cursan con episodios de reagudización que se superponen a la inflamación ya presente. Idealmente, el tratamiento de enfermedades inflamatorias crónicas debería transcurrir en paralelo a la propia enfermedad en sí, con el fin de ajustarse a las condiciones patológicas variables y prevenir efectos secundarios no deseados. Un sistema de vectores ideal sería aquel que estuviese regulado por la propia enfermedad y que expresara altos niveles de agentes anti-inflamatorios durante los episodios recurrentes y niveles muy bajos durante las fases de remisión de la enfermedad.

Hasta el momento se han descrito varios sistemas de expresión viral que responden a estímulos inflamatorios *in vivo* (van de Loo et al, 2004). Sin embargo, tanto la inducción de una respuesta inmune como la rápida pérdida de la expresión del transgén disminuyen la eficacia terapéutica de estos vectores. Los AAV son candidatos prometedores para una terapia génica a largo plazo, ya que se integran en zonas concretas del genoma del huésped y no están involucrados en ninguna patología humana conocida; sin embargo, se ha demostrado recientemente que en determinadas circunstancias pueden provocar una respuesta inflamatoria en el huésped (Mingozzi and High, 2007; Zaiss et al, 2008; Peden et al, 2009). Los vectores lentivirales parecen vectores más apropiados para su uso en procesos inflamatorios crónicos, ya que pueden infectar tanto células en división como en reposo,

proporcionan una expresión a largo plazo, y se caracterizan por una baja inmunogenicidad (Buchschacher and Wong-Staal, 2000).

En la actualidad, los promotores virales son los más utilizados para la expresión de transgenes, pero estos promotores se suelen silenciar tras la integración en el genoma y, como resultado, la expresión del transgén *in vivo* es transitoria. Además, el mantenimiento constante de altos niveles de moléculas anti-inflamatorias aumenta el riesgo de infección, como ya se ha observado en el tratamiento con anti-TNF-α y anti-IL-1 en pacientes con artritis reumatoide (Roth et al, 2002; Mayordomo et al, 2002; Nuñez Martinez et al, 2001; Sicotte and Voskhul, 2001). Asímismo, podría haber una respuesta adaptativa a la concentración constante y elevada de dichas moléculas anti-inflamatorioas, contrarrestando su efecto terapéutico. Se han descrito varios vectores que contienen promotores cuya actividad está controlada por fármacos, consiguiendo una expresión regulada del transgen. Aún así, su mayor inconveniente es la necesidad de una monitorización constante de la enfermedad para determinar las pautas de administración del fármaco para una eficacia óptima; este aspecto se complica aún más por el carácter impredecible y recurrente del curso clínico de la enfermedad.

Introduction

1. CARDIOVASCULAR DISEASES: THE CONCERN OF THE CENTURY

The term *cardio* comes from the Greek καρδιά, *kardia*, meaning *heart.* The term vascular comes from latin uās, meaning duct, and -cul(um), meaning small. In the term cardiovascular, it refers to *blood vessel*s which play a pivotal role in virtually every medical condition. Cancer, for example, cannot progress unless the tumor causes angiogenesis (formation of new blood vessels from pre-existing ones). Blood vessel permeability is increased in inflammation. Damage, due to trauma or spontaneously, may lead to haemorrhage due to mechanical damage to the vessel endothelium. In contrast, occlusion of the blood vessel by atherosclerotic plaque, by an embolised blood clot or a foreign body, leads to downstream ischemia (insufficient blood supply) and possibly necrosis. We also know what a cardiovascular disease (CVD) is, which are the risk factors, which are the healthy habits that can prevent them, which drugs are needed to be taken when suffering from them.

With such a broad knowledge about CVDs, it is hard to believe that they currently are the leading cause of death worldwide, and its prevalence likely to increase in the near future. As a matter of fact, the morbidity and mortality associated with CVD causes an enormous economic burden, which has become a major problem for many societies across the globe. Numerous contributing factors that trigger CVDs have been identified, and many of them are a function of human behavior. Energy balance has shifted in individuals in much of the population when compared to prior generations, such that energy consumption is out of proportion to energy expenditure. This modern lifestyle that includes lack of exercise, consumption of products high in calories and fat, and smoking results in obesity, high cholesterol, diabetes, and high blood pressure (Fuster and Vahl, 2010). Given the increasing prevalence of this disease, efforts should be refocused on the treatment and prevention of the underlying risk factors of CVDs, a strategy that is both easier and less costly than treating the advanced disease.

2. ATHEROSCLEROSIS: A CHRONIC INFLAMMATORY CVD

Atherosclerosis is the most common cardiovascular disease and the main cause of death in the Western world, characterized by the thickening and hardening of the vessel wall. It was once viewed simply as a disorder of inappropriate lipid deposition in the arterial wall, which would ultimately lead to significant narrowing of the artery with subsequent hemodynamic compromise. Today, however, it is widely studied that most of the myocardial infarctions originate from ruptures of plaques that had not had come out by conventional angiography as significantly (Fuster and Vahl, 2010).

Atherosclerosis is characterized by the thickening of the arterial wall that will end up in the formation of an atherosclerotic plaque. In this process cholesterol deposition, inflammation, extracellular-matrix formation and thrombosis play important roles.
Symptoms occur late in the course of disease and are caused in most cases by the narrowing of the lumen of the artery, which can happen gradually as the plaque forms or all of a sudden, whenever a plaque ruptures and there is a thrombotic event. The resultant decrease in blood supply can affect most organs, being coronary heart disease and stroke the most common consequences (Libby, 2010).

Over the past few years an understanding of the importance of inflammation during all stages of atherosclerosis, from its inception through its progression and its final complication of thrombosis, has greatly increased. No longer do we view atherosclerosis merely as a cholesterol storage disease that obstructs arteries. Our therapeutic goals now reach beyond addressing flow-limiting stenoses by invasive revascularization procedures. Much of the plaque's peril lies in its thrombogenic potential, not just the degree of stenosis it causes.

2.1. THE ROLE OF THE VASCULAR ENDOTHELIUM IN ATHEROSCLEROSIS

The former work has highlighted the role of the endothelial cell in endogenous protection against atherosclerosis and as a central cell in the formation of atherosclerotic plaques and their complications. It can also be related to this disease as a key target for an anti-atherosclerotic therapy.

The normal endothelium possesses a variety of properties that actively maintain vascular homeostasis, such as haemocompatibility, regulation of the vascular tone and anti-inflammatory actions. The endothelial cells (ECs) that line the inner layer of the vessel wall, known as the intima layer, are constantly in contact with blood without triggering thrombosis. Early studies in experimental atherosclerosis revealed adhesion of leucocytes to the arterial intima endothelial layer as an early morphological alteration during atherogenesis (Libby et al, 2006a).

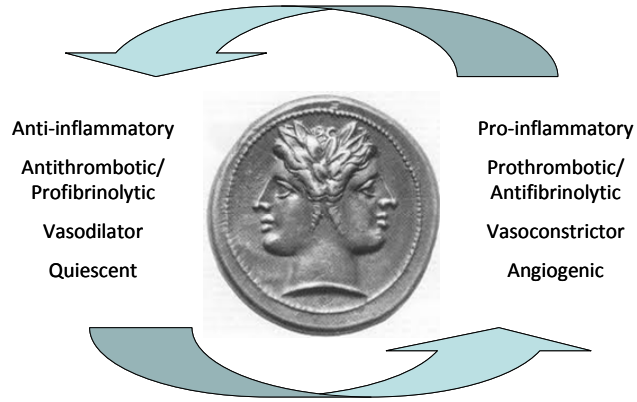

Figure 1. The two faces of the endothelial cell. Like Janus**,** the Roman god of thresholds, of beginnings and transitions, depicted as having two heads, facing opposite directions, just like the two faces of the endothelial cell. Strategically located at the interface of blood and tissues, the EC must look in both directions. The duality between the hemostatic properties (left) and the functions involving the host defence, tissue injury and other diseases (right) also evokes the two faces of Janus. *Adapted from Libby et al, 2006a.*

Several studies during the 80´s unravelling the endothelial adhesion biology led to the identification of several selective adhesion molecules, including vascular adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), P- and E-Selectins, which are expressed on the surface of vascular endothelial cells and mediate this capture of blood leukocytes. The expression of both VCAM-1 and ICAM-1 are upregulated in atherosclerosis, especially in the initial stages. Selectins are carbohydrate-binding molecules that bind to glycoprotein ligands, and are expressed by ECs, leukocytes and platelets. During early stages of the plaque formation, selectins are expressed by the activated endothelium and will mediate the rolling interaction of blood leukocytes with ECs (Kampoli et al, 2009; Ley, 2003).

Vascular disease is a pathological state of large and medium sized arteries and is triggered by EC dysfunction. Because of factors like pathogens, oxidized low density

lipoproteins (ox-LDL) particles and other inflammatory stimuli ECs become activated. This leads to a change in their characteristics: ECs start to secrete cytokines and chemokines and express adhesion molecules on their surface which result in the recruitment of white blood cells (monocytes and lymphocytes), which can infiltrate the blood vessel wall. Stimulation of smooth muscle cell layer with cytokines produced by ECs and recruited white blood cells causes smooth muscle cells to proliferate and migrate towards the blood vessel lumen. The process causes thickening of the vessel wall, forming a plaque consisting of proliferating smooth muscle cells (SMC), macrophages and various types of lymphocytes. This plaque results in obstructed blood flow leading to diminished amounts of oxygen and nutrients that reach the target organ. In the final stages, the plaque may also rupture causing the formation of clots, and as a result, strokes.

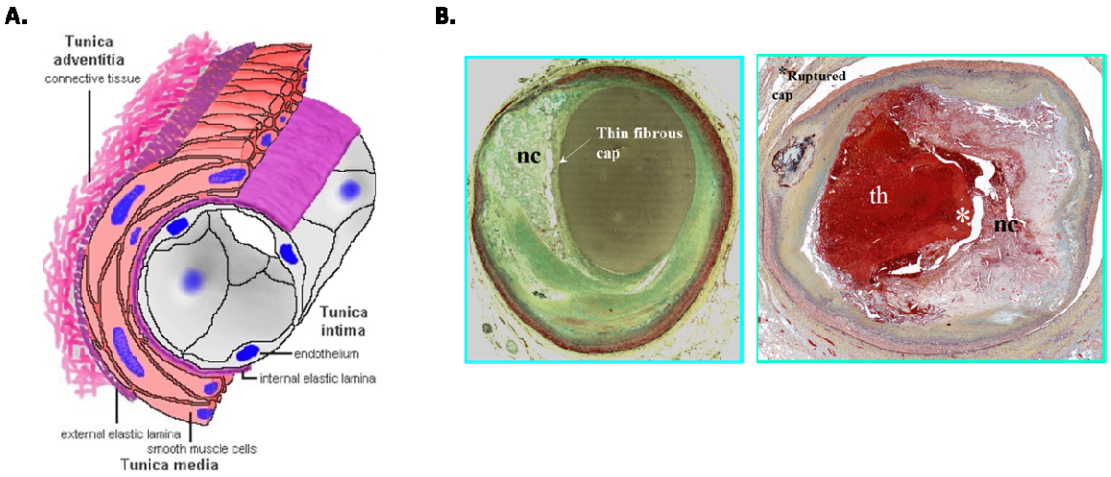

Figure 2. Pathology of the vessels. (A) Schematic diagram of a functional vessel wall showing its different layers, from the inner endothelium to the outer adventitia**. (B)** Cross-sections of an atherosclerostic vessel with a vulnerable plaque (left pictogram) characterized by a thin fibrous cap and a necrotic core (nc) that ends in the rupture of the plaque (right pictogram) and a thrombus formation (th) that ocludes the vessel lumen. *Pictures courtesy of Dr Badimon.*

2.2. INITIATION AND DEVELOPMENT OF ATHEROSCLEROTIC LESIONS

Inflammatory processes govern many of the aspects of the biology of plaques that determine their clinical destiny. Under normal conditions, the ECs of the arterial wall resist adhesion and aggregation of leukocytes and promote fibrinolysis (Libby, 2010). However, well-known risk factors of atherosclerosis, such as hypertension, hyperlipidemia, smoking, hyperglycemia, insulin resistance, and obesity, can trigger the expression of adhesion molecules that enable the attachment of leukocytes to the endothelial layer of the arterial wall. Leukocyte *rolling* along the endothelial surface is the first step in leukocyte adhesion to endothelial cells. Several studies have demonstrated that this leukocyte rolling is mediated in the first place by the selectin family of adhesion receptors and their carbohydrate ligands (Dong et al, 1998). L-selectin is constitutively expressed on the surface of most leukocytes; E-selectin is found exclusively on ECs activated by inflammatory cytokines; P-selectin is rapidly released from storage granules in activated platelets and ECs. The generation of selectin-deficient mice has confirmed the role of selectins in the rolling of blood cells and has provided evidence indicating that there is an overlapping function of these selectins in the regulation of inflammatory processes (Ley, 2003).

VCAM-1 is the next key element in this process. Monocytes and lymphocytes interact with EC through selectins and VCAM-1 and later migrate into the developing atherosclerotic site (Libby, 2006b). After adherence, chemokines promote the penetration of leukocytes into the vessel wall. Monocyte chemoattractant protein-1 (MCP-1) is one of the molecules identified as a key mediator of the passage through the unruptured blood vessel wall or diapedesis of monocytes into the intima of arteries (Hansson, 2005; Hansson and Hermansson, 2011). Monocytes that have penetrated the arterial wall express scavenger receptors for modified lipoproteins, consume lipids, and transform into foam cells. Macrophage stimulating factor (M-CSF) is a monocyte activator and a key mediator of this transformation process. In parallel, macrophages proliferate and amplify the inflammatory response through the secretion of numerous growth factors and cytokines, including tumor necrosis factor (TNF) and interleukin 1 (IL-1).

T-lymphocytes also play a major role in the inflammatory process within the arterial wall through production of cytokines, such as interferon-gamma (IFN-γ) and TNF-α and -β. This inflammatory process progresses through cytokine-stimulated activation of leukocytes, release of fibrogenic mediators, and replication of smooth muscle cells (SMCs), leading to more complex atherosclerotic lesions (Packard and Libby, 2008).

2.3. PROGRESSION TO COMPLEX ATHEROSCLEROTIC LESIONS

Macrophages and T cells infiltrate atherosclerotic lesions and localize in the *shoulder* region, which is the region where the atheroma grows. Whereas foam cell accumulation characterizes fatty streaks, deposition of fibrous tissue defines the more advanced atherosclerotic lesion. Vascular Smooth Muscle Cells (VSMCs) synthesize the bulk of the extracellular matrix that characterizes this phase of plaque evolution (Libby, 2006b).

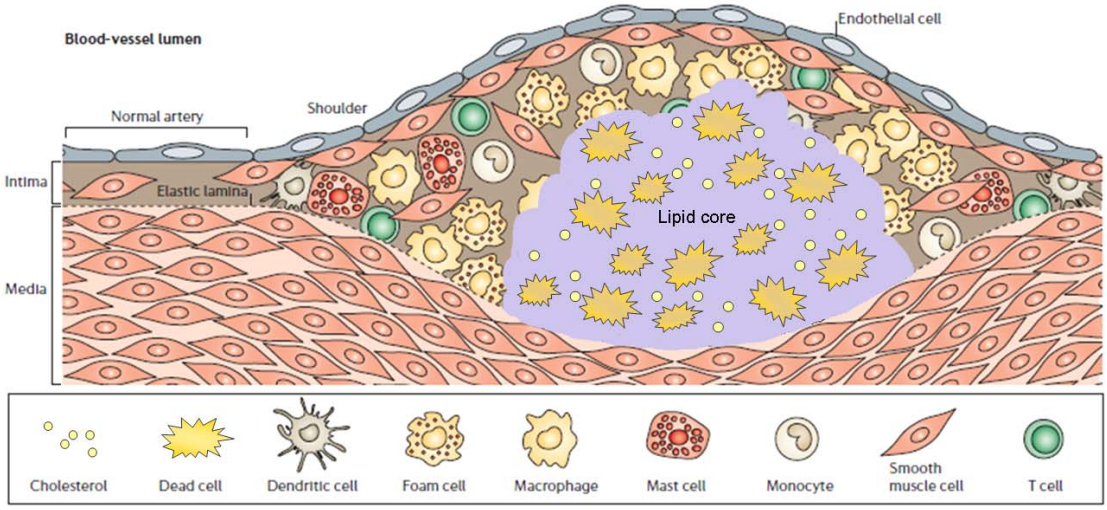

Figure 3. Cellular composition of atherosclerotic plaques. The atherosclerotic plaque has a lipid core that contains esterified cholesterol, cholesterol crystals and debris from dead cells. Around it, a fibrous cap of smooth muscle cells and collagen helps to stabilize the plaque. Immune cells such as macrophages, T cells and mast cells inside the plaque are usually in an activated state. They produce cytokines, proteases and pro-thrombotic molecules which contribute to the inflammation in the plaque and affect the vascular function. *Adapted from Hansonn and Libby, 2006.*

In response to platelet-derived growth factor (PDGF) released by activated macrophages and ECs, and silent plaque disruptions that lead to clinically unapparent mural thrombi, VSMCs migrate from the tunica media into the intima via degradation of the extracellular matrix mediated by Matrix Metallopeptidase 9 (MMP-9) and other

proteases. In the intima, VSMCs proliferate under the influence of various growth factors and secrete extracellular matrix proteins, including interstitial collagen, especially in response to transforming growth factor (TGF) and PDGF. This process causes the lesion to evolve from a lipid-rich plaque to a fibrotic and, ultimately, a calcified plaque that may create an abnormal narrowing of the blood vessel or stenosis (Packard and Libby, 2008).

Neovascularization arising from the artery's network of small blood vessels (known as vasa vasorum) contributes to lesion progression in many ways and it provides another portal for leukocyte entry into established atherosclerotic lesions. In addition, these fragile neovessels can favor focal intraplaque hemorrhage which provides a mechanism for the discontinuous increments seen in plaque growth. Local hemorrhage within the plaque in turn generates thrombin, which activates ECs, monocytes/macrophages, VSMCs, and platelets. These molecules further promote lesion formation and favor the thrombotic complications of atherosclerosis (Libby, 2010).

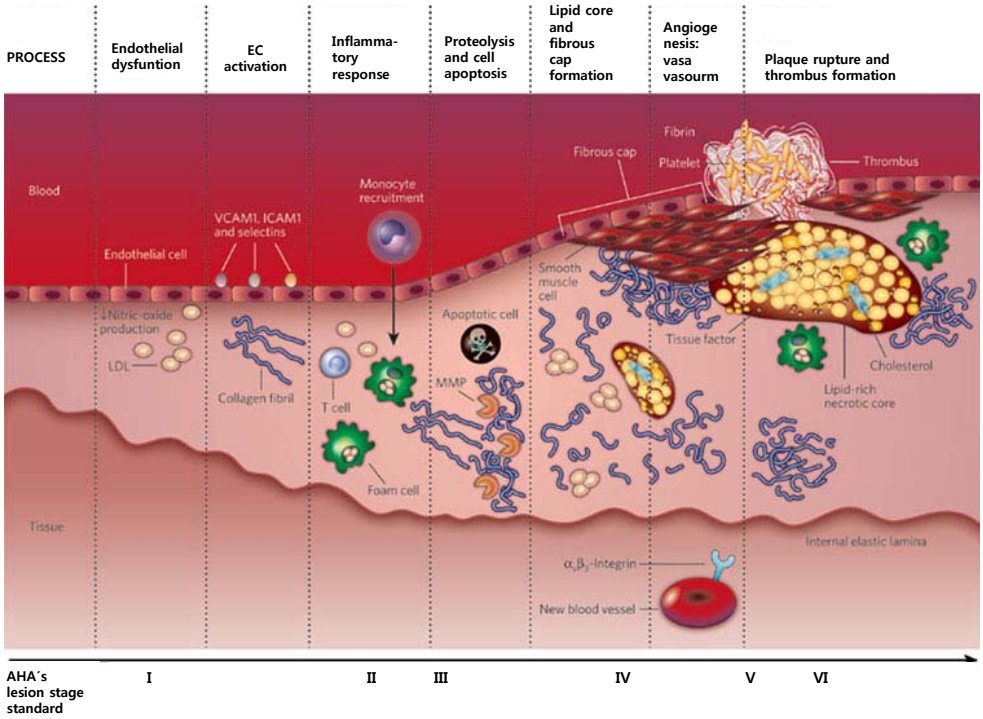

Figure 4. Schematic representation of the development of atherosclerotic lesions. This graph depicts a simplified scheme of the development of atherosclerotic plaques starting with a normal blood vessel (left) progressing to a vessel with a complex atherosclerotic plaque with luminal thrombus formation (right) (AHA, American Heart Association; ICAM1, Intercellular adhesion molecule 1; MMP, matrix metalloproteinase; VCAM1, vascular cell-adhesion molecule 1). *Figure adapted from Sanz and Fayad, 2008.*

2.4. ANIMAL MODELS FOR THE STUDY OF ATHEROSCLEROSIS

Numerous animal species have been used to study the pathogenesis and potential treatment of atherosclerosis. The first experimental model appeared back in 1908 when it was reported the formation of atherosclerotic lesions in aortas of rabbits fed with a diet rich in animal proteins (Jawien et al., 2004). The study of atherosclerosis has been restricted for a long time to large animals such as swine, rabbits or non-human primates. Rabbits do not develop spontaneous atherosclerosis but are highly responsive to cholesterol-rich diets and develop lesions in a short time. These lesions though, are more macrophage–rich than the human ones and cholesterol levels in plasma are extremely high. The pig is a very good model as they reach plasma cholesterol levels and lesions much more similar to humans when fed on a high cholesterol diet. Although these large models have provided with invaluable insights of this disease, the cost of maintenance and handling of the colonies is very high. The use of murine models has implied a breakthrough in the study of the atherosclerotic disease.

2.4.1. Murine Models for the Study of Atherosclerosis

The mouse as a model of experimental atherosclerosis meets many of the criteria desired for a useful animal model: it is easy to handle, easy to breed a large colony and can develop atherosclerosis in a very short timeframe. Mice are very resistant to atherosclerosis except for the C57BL/6 strain, which develop lesions when fed on a high cholesterol diet. The first studies on this animal model where made during the 1960´s, and Paigen and collaborators developed the *Paigen diet,* a diet rich in fat and cholesterol and a low percentage of cholic acid. This model was widely used although its main disadvantage was that after feeding the animals for 14 weeks to 9 months periods the lesions obtained were small, mainly localized in the aortic root and differed in composition to the human lesions. Therefore, the creation of new models genetically manipulated to modify the progression of the disease was a real breakthrough.

2.4.1.1. About the apoE Mouse Model

The apolipoprotein E-deficient mice (apoE mice) are mice that have had the APOE gene disrupted by replacing part of gene with a neomycin-resistant gene (Piedrahita et al, 1992); therefore these animals are devoid of apoE protein which plays a central role in lipoprotein metabolism. ApoE is also required for the efficient clearance of diet-derived chylomicrons and liver-derived VLDL (very low density lipoprotein) remnants by the liver. Consequently, this model provided the first practical model of hyperlipidemia and atherosclerosis. Being atherosclerosis mainly a human disease, and given the existing differences between mice and men, it was never thought that a mouse model could be used to mimic this human disease. But after years of working with mice, the apoE mice has turned out as a reliable model for the study of human-like plaques, providing a platform to study in detail the course of the disease, as well as exploring different therapies (Avani et al, 2009).

ApoE mice are considered to be one of the most relevant models for atherosclerosis because of their high hypercholesterolemia and development of spontaneous arterial lesions, similar to those in humans. This process can be accelerated with the administration of a diet rich in cholesterol. Hayek and collaborators developed a more physiological than Paigen´s diet called the *Western diet,* based on the American diet of several years ago. On this diet, lesion formation is greatly accelerated and lesion size is increased (Figure 5) (Plump and Breslow, 1995; Jaiwen et al, 2004). *The* atherosclerotic plaques are formed systematically in the most vulnerable areas of the large vessels, the aortic root and the aortic arch, areas where the blood flow is more turbulent (Crauwels et al, 2003). Therefore, this model is considered a real breakthrough as it has been used to identify atherosclerosis susceptibility modifying genes and the role of various cell types in atherogenesis, associate environmental factors affecting atherogenesis, and assess therapies that might block atherogenesis or lesion progression (Jawien et al, 2004).

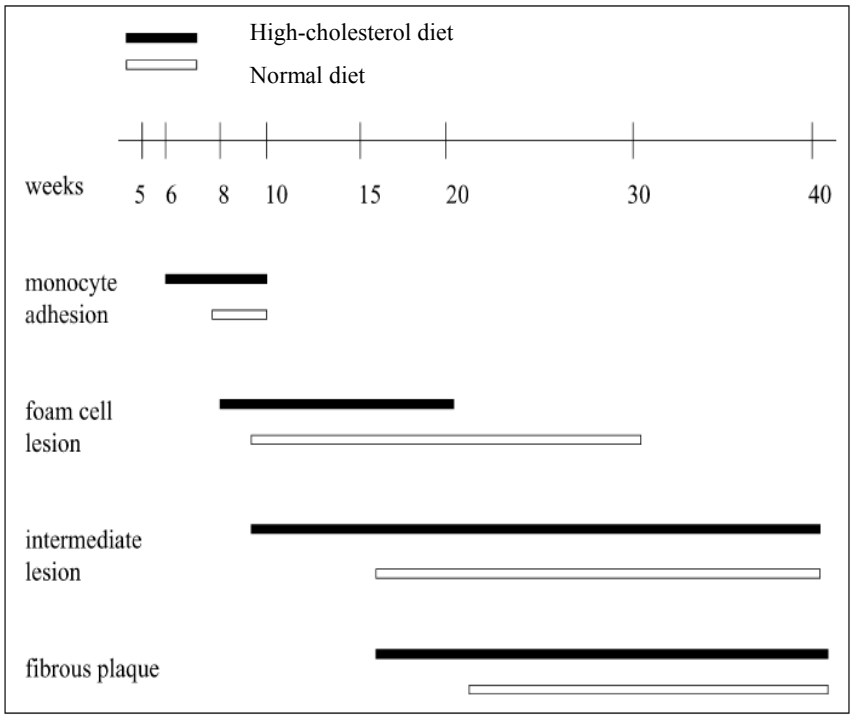

Figure 5. Atherosclerotic lesion progression in the ApoE mouse model. Diagram showing how lesion formation in mice on a high-cholesterol diet (aka Western-type diet) is accelerated in comparison with mice on a normal diet. *Adapted from Jawien et al, 2004.*

2.4.1.2. Other Atherosclerosis Mouse Models

The LDL receptor-knockout (LDLR-KO) mice were created as a model of familial hypercholesterolemia. They have a high increase in LDL and VLDL on a normal diet without developing atherosclerosis, but do have a high response to a Paigen diet, that leads to high plasma cholesterol levels and development of large lesions. It is not as well characterized as the apoE mice model, but the lesion composition and progression in these mice are very similar to those found in apoE mice and, therefore, in human lesions. Another recently developed model, the double KO for APOE and LDLR (APOE/LDLR-DKO) mice, develops hypercholesterolemia and atherosclerosis with a more marked development of atherosclerosis than apoE mice on a normal diet (Jawien et al, 2004).

In conclusion, gene-targeted mouse models have changed the face of atherosclerotic research and helped out with the definition of atherosclerosis as an inflammatory disease.

3. THE PHOSPHATASE CALCINEURIN

3.1. THE PROTEIN

Calcineurin (CN) was purified in the late 1970s by Dr. Claude Klee and named due to its regulation by calcium and its enrichment in neural tissues (Klee, 1979). CN is a unique calcium/calmodulin-activated serine/threonine phosphatase that mediates calcium-dependent signal transduction pathways in eukaryotes, most notably through nuclear factor of activated T-cells (NFAT). CN is involved in T-cell activation, cytokine synthesis, skeletal and cardiac muscle growth and differentiation, memory processes, and apoptosis of T-lymphocytes, ECs, neuronal cells, and macrophages. It is also known to mediate neurotransmitter activity in the brain, where it is constitutes 41% of the total brain protein (Aramburu et al, 2000).

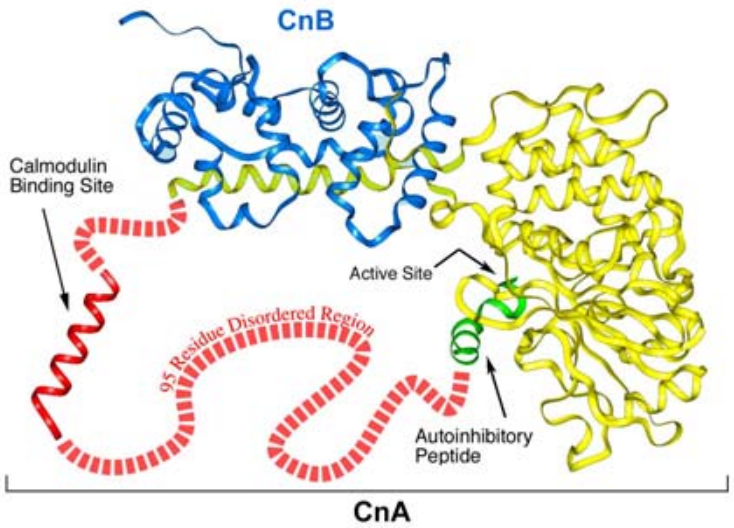

Figure 6. Structure of Calcineurin. Ribbon representation of the secondary structure of calcineurin regulatory subunit or CnB (in blue) non-covalently bound to calcineurin catalytic subunit or CnA. This latter subunit contains the phosphatase domain (in yellow) and the autoinhibitory domain (in green) which are connected by a disordered region (in red). It is speculated that the Calmodulin binding site turns into an alpha-helix in the presence of Calmodulin.

CN is a heterodimer comprising a catalytic subunit A (CnA) and a regulatory subunit B (CnB). CnA contains the phosphatase domain and a regulatory region, which are connected by a 13 amino acid linker sequence. The regulatory region contains a CnB-binding domain (BBD), a calmodulin-binding domain, and an auto-inhibitory domain (AID) (Klee et al, 1998). CnB contains four EF-hand calcium-binding motifs and tightly binds to CnA at sub-micromolar concentrations of calcium. At low calcium

concentrations, calmodulin (CaM) is not bound to the complex, and the AID sterically blocks the active site. As calcium concentration rises, calcium binds the EF-hand motifs of CaM, allowing its interaction with CnA and, as a consequence, a conformational shift so that the AID no longer obstructs the active site (Rusnak and Mertz, 2000).

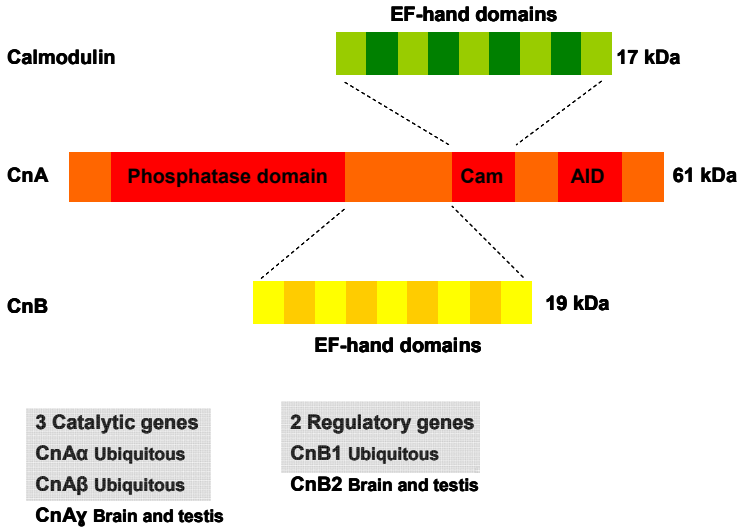

Figure 7. Schematic representation of the catalytic calcineurin A subunit (CnA), the regulatory calcineurin B subunit (CnB), and calmodulin. Three calcineurin A genes and two calcineurin B genes encode for the corresponding calcineurin subunits (grey boxed the isoforms that contribute to calcineurin activity in most vertebrate tissues). Cam, calmodulin-binding domain; AID, autoinhibitory domain. *Adapted from Wilkins and Molkentin, 2004.*

3.2. *CALCINEURIN/NFAT PATHWAY*

CN is activated by an increase of calcium in the cytosol, and in turn dephosphorylates a number of cellular substrates including NFAT (Crabtree, 1999). Proteins of the NFAT family (NFATc1-c4) are transcription factors that regulate gene transcription in response to intracellular calcium signals. Their presence in the tissues is quite broad, including the endothelium, skeletal and smooth muscles, the myocardium and the brain. When NFAT is not active it is localized in the cytoplasm and it is highly phosphorylated. Whenever CN is activated by intracellular calcium signals, it dephosphorylates NFAT inducing its translocation to the nucleus. Once in the nucleus NFAT activates the transcription of numerous genes including the cytokine and immune system regulators interleukin-2 (IL-2), IL-3, IL-4, IL-5, TNF-α, granulocyte macrophage colony-stimulating factor (GM-CSF), IL-12 p40, IL-2 receptor; CD40L, FasL, and CD25 (Rao, 1997).

In order to control gene transcription, NFAT binds DNA and interacts with other transcription factors (e.g. AP-1, MEF-2 or GATA-4); these interactions may also stabilize its nuclear localization. Activity of nuclear NFAT is terminated by intracellular kinases (e.g. p38, JNK, GSK3) and rephosphorylation of NFAT results in transport back to the cytoplasm via an export pathway that involves the transport protein Crm1 (exportin 1) (Gomez del Arco et al, 2000).

The dephosphorylation of NFAT requires a previous interaction between NFAT and CN proteins; they physically interact through two different identified binding sites (Liu et al, 2009; Li et al, 2011). Aramburu and collaborators revealed the existence of an important CN-binding site, conserved among the four NFATc1-c4 members and located near the N-terminus of the NFAT regulatory domain which contains the consensus sequence ‘‘PxIxIT’’ (‘‘x’’ denotes any amino acid). Interfering with the docking of CN at the PxIxIT sequence impairs NFAT activation and NFAT-dependent reporter gene expression (Aramburu et al, 1999). A high affinity peptide derived from the PxIxIT sequence, known as VIVIT, allowed the identification of the PxIxIT docking site on CN; it was shown that VIVIT forms contacts along the CnA phosphatase domain (Li et al, 2004). The VIVIT peptide was more effective than the original peptide at inhibiting CN-mediated dephosphorylation of NFAT in vitro without disrupting other CN-dependent pathways (Aramburu et al, 1999).

In addition to the PxIxIT motif, a second CN-binding motif was identified which lies near the C-terminus of the NFAT regulatory region (Liu et al, 1999; Liu et al, 2001; Park et al, 2000; Martinez-Martinez et al, 2006). This motif contains a consensus sequence LxVP and, together with PxIxIT, contributes to the overall affinity of the substrate for CN; variation in the LxVP sequence modifies the binding of the substrate to the phosphatase (Martinez-Martinez et al, 2006). Unlike the PxIxIT motif, a peptide based on the LxVP sequence inhibits the CN phosphatase activity and requires CnA bound to CnB for the interaction. It has been shown that the LxVP peptide binds to a hydrophobic pocket at the interface of the two CN subunits (Rodriguez et al, 2009). This CN hydrophobic docking surface formed at the junction of CnA and CnB is also essential for binding of the immunosuppressant drugs cyclosporin A (CsA) and FK506,

which are important for post transplant recovery treatments (Griffith et al, 1995; Huai et al, 2002; Jin and Harrison, 2002; Kissinger et al, 1995). In contrast with CsA and FK506 that need to form complexes with specific immunophilin binding proteins to bind to CN, the LxVP peptide interacts directly with the described CN docking surface (Rodriguez et al, 2009).

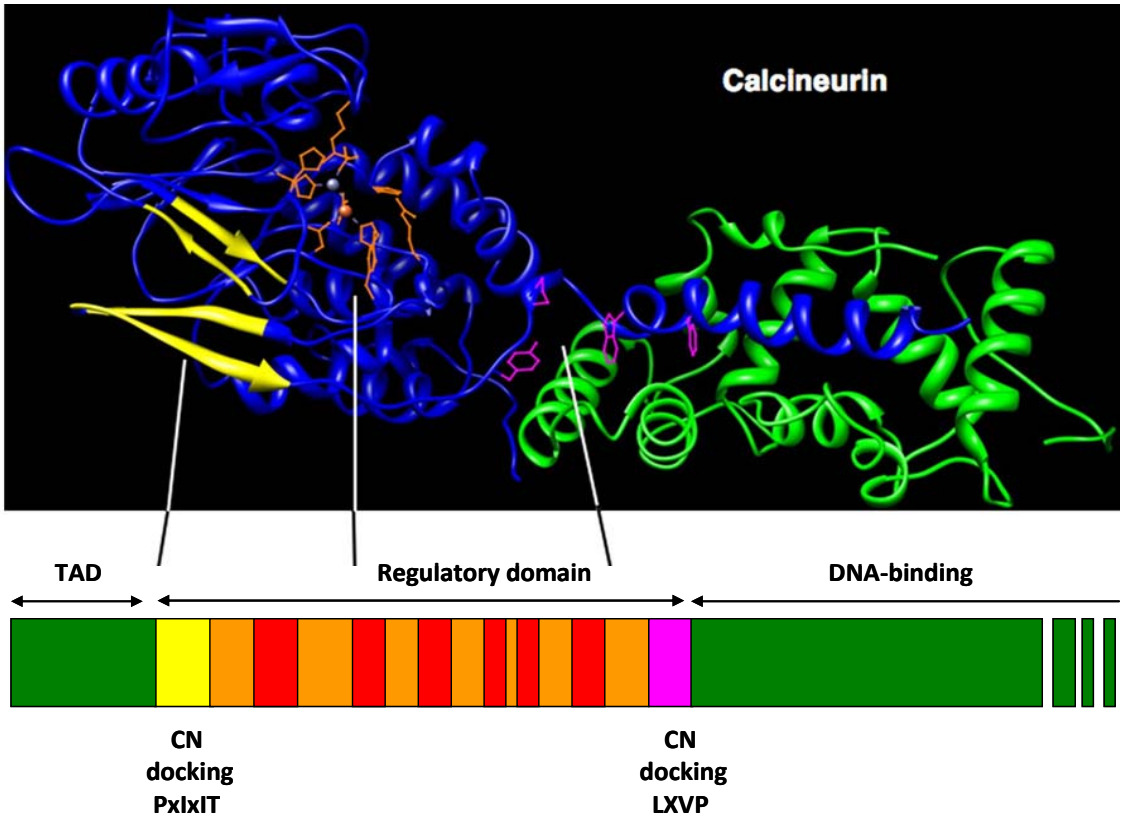

Figure 8. Secondary structure of calcineurin and the NFAT domains. (Top) Secondary structure of CnA (blue) and CnB (green). The PxIxIT binding site (yellow), the active site (orange) and the LxVP binding site (pink) are indicated. (Bottom) Schematic representation of the NFAT domains: the transactivation (TAD; N-terminus) and DNA-binding (C-terminus) domains are shown in green. The regulatory domain contains the phosphorylation sites (red) surrounded by the PxIxIT (yellow) and LxVP (pink) sequences. *Adapted from Liu et al, 2009; Li et al, 2011.*

3.3 CN/NFAT Pathway and CVDs

Five members of NFAT (NFATc1 to NFATc5) have been cloned to date, with NFATc1 to NFATc4 being expressed in the cardiovascular system. The NFATc3 and NFATc4 members are active during pathological conditions that affect the cardiovascular system, including atrial fibrillation and hypertrophy. Many studies have identified alterations in calcium handling in the failed myocardium such that the amplitude of the intracellular calcium transient is decreased and prolonged. These studies have

suggested the hypothesis that alterations in intracellular calcium handling progressively exacerbate a hypertrophic or cardiomyopathic phenotype, in part, through sustained activation of calcium-sensitive signal transduction pathways. The recent identification of CN as a hypertrophic regulatory factor supports such a hypothesis (Molkentin, 2000).

Despite having studied intensively the role of the CN/NFAT-dependent pathway in the development of cardiac hypertrophy, much less is known about its role in blood vessels or its possible implication in atherosclerosis or other cardiovascular diseases (Wilkins et al, 2004). In the vascular system, members of NFAT (c1 and c3) are implicated in cell growth, remodeling of SMCs, control of vascular development and angiogenesis; NFATs are also activated in response to inflammatory processes and high intravascular pressure. In the endothelium, NFATs control gene expression during remodeling and are activated by growth factors (Hernandez, 2001; Boss, 1998). However, the precise physiological or pathological calcium signals that cause activation of the CN/NFAT pathway in endothelial cells have not yet been determined (Rinne et al, 2009). The critical role played by CN in the regulation of T-cell reactivity and cytokine gene expression, as well as its implications in the calcium-dependent pathways, led us to hypothezise about its major implications in cardiovascular diseases such as atherosclerosis or restenosis.

4. CALCINEURIN AS A THERAPEUTIC TARGET

CN acts as a multifunctional regulator of various downstream targets, such as NFAT, NF-*κ*B, myocyte enhancer factor-2 (MEF2), and the apoptotic factor Bad. NFAT was initially identified in T lymphocytes as an essential regulator of IL-2 expression (Shaw et al, 1988). Further on, NFAT was also implicated in osteoclast differentiation, muscle fiber-type specialization, cardiac valve and myocardial development. CN activates NFAT not only in T cells, but also in B-, natural killer-, and mast cells as well as all major vascular cell types including VSMCs, ECs, and macrophages. These facts highlight NFAT as an important modulator in the development and function of the cardiovascular system (Yu, 2007).

CN/NFAT signaling has emerged as a crucial therapeutic target in the regulation of inflammation and hyperplasia of the myocardium and VSMCs. Selective NFAT inhibition is seen to be beneficial in both cardiopathy and vasculopathy (Yu, 2007). Given the crucial role of CN signalling in various physiological and immunological processes, its inhibition is considered a powerful therapeutic modality in the treatment of graft transplant rejection, autoimmune diseases, and cardiovascular disorders.

4.1. PHARMACOLOGICAL INHIBITION

CN is the target of the immunosuppressants CsA and FK506 (aka Tacrolimus), which as suppressors of graft rejection are important treatments for post-transplant recovery (Liu, 1991). As investigative tools, CsA and FK506 have enabled roles of CN to be identified in diverse biological processes, including cell-stress responses and drug tolerance in fungi; in mammals, CN is involved in immune responses, pancreatic beta cell function, early vascular development, neurological functions, ion homeostasis, and muscle differentiation. In addition to their use in preventing transplant rejection, CN inhibitors have been successfully used to treat atopic dermatitis, severe asthma, segmental glomerulosclerosis, and refractory rheumatoid arthritis (Reynolds, 2002; Powell, 2001; Cattran, 2007; Suzuki, 2010).

CN activity is inhibited by CsA and FK506. The way these two molecules bind to CN is by each forming complexes with a specific immunophilin binding protein (cyclophilin [Cyp] and FK506 Binding Protein [FKBP], respectively). Immunophilins (IPs) bind CN only in the presence of immunosuppressants (IS); IPs are prolyl isomerases, but this activity appears to be unrelated to immunosuppression. Several structural, biochemical, and genetic studies have shown that immunosuppressant-immunophilin (IS-IP) complexes interact with a hydrophobic surface formed at the junction of the two CN subunits. A small set of amino acids in CN is involved in the recognition of both IS-IP complexes (Ke and Huai, 2003). Since CsA (a cyclic peptide) and FK506 (a macrolide) are structurally different, their interaction with the same residues in a common site in CN suggests that this site can be a recognition region for other CN-binding proteins (Rodriguez et al, 2009).

4.1.1. Cyclosporine A (CsA)

CsA is a fungal cyclic undecapeptide discovered in the early seventies, commonly used in organ transplant recipients and in patients with autoimmune disorders (Wenger and Payne, 1989). CsA was originally isolated from cultures of the fungus *Tolypocladium inflatum* grown from a soil sample collected in Southern Norway. CsA was quickly demonstrated to be effective in a wide variety of animal models of transplantation and autoimmunity. The introduction of CsA in 1983 for the treatment of renal allograft rejection had a profound impact on the field of organ transplantation.

CsA treatment is associated with various side effects, including nephrotoxicity, diabetes, and hypertension, which have recently been reviewed (Robert et al, 2010). The major toxicity of CsA in animal models is nephrotoxicity, manifested by decreased glomerular filtration rates, associated with a reduction in renal blood flow that reflects an increase in vascular resistance. The vasoconstriction caused by CsA is not confined to just the kidney but is found also in the systemic circulation.

Importantly, CsA has also been associated with increased susceptibility to atherosclerosis and the development of hyperlipidemia. Many of the cells and intracellular pathways relevant to atherosclerosis are likely to be affected by CsA. The studies reviewed here show that CsA affects multiple signalling molecules thereby affecting lipid, secretory and inflammatory pathways in SMCs, macrophages, endothelial, and T cells. Although immunosuppression by CsA might be expected to be anti-atherogenic, the diversity of the pathways affected does not allow simple conclusions to be drawn (Kockx et al, 2010).

Although very effective as immunosuppressant, CsA also affects many cellular pathways not associated with immunosuppression, several of which can be linked to the cardiovascular side effects observed in transplant recipients. CsA treatment causes complex effects on lipoprotein metabolism and bile acid production; it also affects EC, VSMC and macrophages, all of which are critical to the atherosclerotic process. In ECs it inhibits the transcriptional activity of NFAT, blocking Vascular Endothelial Growth Factor-A induced signalling, therefore inhibiting their proliferation and migration. Several studies, like the ones made by Satonaka and collaborators (2004) and Liu and

collaborators (2005), have demonstrated that CsA inhibits proliferation and migration both in vitro and in vivo of VSMC, suggesting that CsA could exhibit anti-atherogenic effects (Kockx et al, 2010). In addition, Angiotensin II-induced expression of MCP-1 by VSMC is suppressed by CsA via post-transcriptional mechanisms, therefore macrophage infiltration could be inhibited, stopping the lesion formation (Satonaka et al, 2004). CsA affects several inflammatory pathways relevant to macrophages, although it is still under study. It inhibits the potential of monocyte/macrophages to trigger autologous and allogeneic mixed lymphocyte reactions as well as inhibiting secretion of apoE from human foam cell macrophages (Kockx et al, 2010).

4.1.2. FK506

The search for safer and more effective immunosuppressants led to the discovery of FK506 in the fermentation broth of *Streptomyces tsukubaensis*, an organism obtained from soil samples collected in the Tsukuba area of northern Japan. In general, FK506 is more potent than CsA in a variety of experimental models of transplantation and autoimmunity; but except for some minor experimental differences, both compounds have a similar spectrum of activity in these models. Studies done in several laboratories, however, indicated that FK506 treatment produced behavioural changes and nephrotoxicity in the rat and severe gastrointestinal toxicity and myocardial damage in the dog, although little pathology was noted in primate studies. With this background of toxicological data, clinical trials with FK506 were begun focusing primarily on liver transplantation (Sigal and Dumont, 1992).

The principal attributes of FK506 include its potency, its ability to slow or to stop progression in the early phases of "chronic" rejection, and its effectiveness in treatment and prevention of acute rejection episodes without resorting to additional immunosuppressive therapies. Use of FK506 permits more rapid tapering of steroids than does CsA therapy, suggesting the potential for its application as mono therapy in other clinical conditions (Sigal and Dumont, 1992).

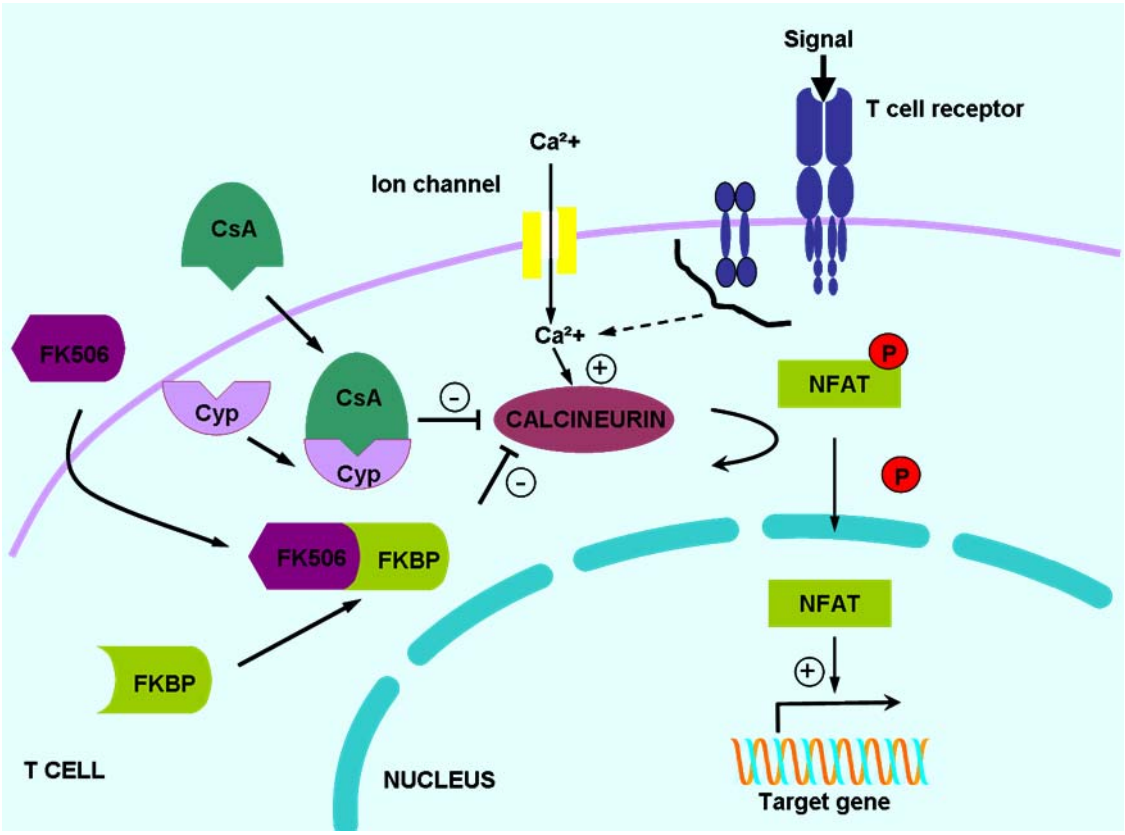

Figure 9. Mechanism of action of cyclosporine A and tacrolimus (FK506). Although CsA and FK506 have unrelated chemical structures, their mechanisms of action, biological effects and clinical side effects are very similar. Given their hydrophobic nature, these compounds diffuse across the plasma membrane, and once in the cytoplasm, CsA and FK506 bind to their immunophilin partners, cyclophilin A (CyP) and FKBP, respectively. The CsA-CyP and FK506-FKBP complexes, but not the unbound drugs, associate with CN and inhibit its phosphatase activity. As a consequence, NFAT proteins stay hyperphosphorylated in the cytoplasm. *Adapted from Sigal and Dumont, 1992.*

4.1.1.1. Immunosuppressants and Atherosclerosis

The link between the atherogenic progression and CN comes from the accelerated atherosclerosis found in renal and kidney transplant recipients. CVDs are responsible for approximately 40% of deaths in renal transplant patients. Also, there is a well established correlation between lipid levels and cardiovascular events in non-transplant populations (Ramezani et al, 2007). Treatment with recommended doses of CsA is associated with nephrotoxicity, resulting in long-term renal dysfunction, hypertension, and hyperlipidemia (Ekberg et al, 2007). The precise role of CsA in causing hyperlipidemia after solid organ transplantation remains unclear as these patients have multiple factors contributing to it, such as post-transplantation obesity and the concurrent use of corticosteroids (Fernandez-Miranda et al, 1998). Randomised studies administrating FK506 or CsA after renal or liver transplantation

have shown hyperlipidemia in patients treated with either agent, being generally more severe in the case of CsA (Ichimaru et al, 2001).

CsA and FK506 inhibit the CN phosphatase activity and their long term administration is associated with a higher risk of atherosclerosis (Apanay, 1994; Sutherland, 1995; Ghanem, 1996; Cofan, 2002; Venkiteswaran, 2001). However, whether the use of these drugs either inhibits or ameliorates the development of transplant atherosclerosis remains unclear, as results obtained in different studies addressing the effect of CsA and FK506 on *de novo* atherosclerosis in animal models are contradictory (Drew, 1995; Matsumoto, 1998; Roselaar, 1995). These differences may be due to unique aspects of each model, but also to inherent CN-independent effects associated with both CsA and FK506. Both drugs bind to IPs, and can alter several biological processes, such as the mitocondrial permeability or the ryanodine receptor function involved in the cellular calcium homeostasis. A recent study shows that low-dose FK506 inhibited collar-induced atherosclerotic plaque progression and stabilized plaques in apoE deficient mice (Donners, 2005). These data suggested that FK506 affects the plaque phenotype at the level of the plaque itself and not through a systemic immunosuppressive effect. As a matter of fact, FK506 shares not only clinical efficacy but also many side effects with CsA, including disturbances in lipid metabolism. Therefore, the prolonged use of these drugs in many chronic diseases other than organ transplantation or atopic dermatitis has been restricted.

4.2. NOVEL CALCINEURIN INHIBITORS: AN ALTERNATIVE TO CURRENT IMMUNOSUPPRESSANTS?

The centrality of CN and the CN/NFAT pathway to immune responses has suggested that interrupting this signaling at any of several points could provide a therapeutic alternative to current immunosuppressive drugs (Kang et al, 2005). As it has already been mentioned in this work, CsA and FK506 disrupt CN phosphatase activity and thus affect all the downstream signal transduction pathways. This rather non-selective inhibition could lead to undesired side effects and toxicity, such as progressive loss of renal function, hypertension and neurotoxicity. It is not yet clear to what extent these toxicities are due to inhibition of NFAT, to interference with dephosphorylation of

other calcineurin substrates, or to potentially calcineurin-independent pathways, such as the up-regulation of transforming growth factor beta (TGF-β) (Aramburu, 1999). For this reason, the development of selective inhibitors (such as small organic molecules and peptides) that inhibit NFAT binding to CN without affecting its catalytic activity would be ideal for proper handling of NFAT-mediated reactions in different diseases (Im and Rao, 2004). NFAT inhibitors with less toxicity than CsA and FK506 could be useful in treating chronic diseases such as myocardial hypertrophy, allergy, arthritis, and autoimmune disease.

4.2.1. Small Organic Molecules

The applications for molecules that bind proteins with high affinity are being intensively studied due to their innumerable bioanalytical research applications in biotechnology and biomedicine. Small organic molecules are prepared by organic synthesis and their molecular weight is lower than in proteins. They are quite efficient as inhibitors of intracellular protein-protein interactions because they have a broad structural diversity that can be exploited to achieve tight binding to the target protein surface. In addition, effective inhibitors can be tailored chemically to improve stability, to eliminate nonspecific side effects, and to enhance delivery *in vivo* (Roehrl et al, 2004). Several Inhibitors of NFAT-CN Association (INCA compounds) have been described which prevent binding of NFAT or the VIVIT peptide to CN (Kang et al, 2005). Although their binding site is located in the phosphatase domain of CnA, it does not coincide with the core PxIxIT-binding site. Therefore the inhibition comes from an allosteric change which has an effect on the PxIxIT docking site. Despite the recent advances in this technology, small organic molecules have had little impact in the field of diagnostics, as the majority of the trials have been cancelled in the last stages for unexpected toxicity and poor efficacy.

4.2.2. CN Inhibitory Peptides

There is considerable evidence suggesting that targeting the CN-NFAT protein-protein interaction may cause a selective inhibition. Several groups have demonstrated that the CN-NFAT interaction is based on recognition of a P*x*I*x*IT and an LxVP sequences in NFAT and that these recognitions are essential for efficient signalling. The recent

development of selective peptides inhibitors of NFAT could be a great perspective in the treatment of cardiovascular disorders.

4.2.2.1. VIVIT

Other than targeting the catalytic site of calcineurin, as effected by CsA and FK506, an alternative strategy was to selectively inhibit the NFAT activation via preventing its protein–protein interaction with CN; NFAT must bind to CN through the described docking sites before it becomes dephosphorylated and activated. The specific interruption of CN docking onto NFAT would allow efficient NFAT inactivation without impairing CN phosphatase activity in general (Yu et al, 2006).

Optimization of the PxIxIT motif through affinity driven selection from a combinatorial peptide library led to the discovery of the VIVIT peptide, which was shown to selectively and potently inhibit CN-NFAT interaction (Aramburu et al, 1999). Until now, no toxic side effects have been reported for VIVIT. Indeed, due to its high specificity to interfere with NFAT signaling with no effect on CN phosphatase activity, the VIVIT peptide is expected to be less toxic than CsA or FK506, whose nephrotoxicity is suggested to be mediated by NFAT independet signaling pathways (Yu et al, 2007). Accordingly with table 1, VIVIT exhibits a much lower toxic profile than CsA and FK506.

Charasteristic	**VIVIT**	**CsA**	**FK506**
Composition	16 member linear L-peptide	Cyclic non-ribosomal undecapeptide	23-member macrolide lactone
M.W.(g/mol)	1684	1203	804
Calcineurin-binding site	Calcineurin docking site on N-terminal regulatory domain of NFAT	Calcineurin active site	Calcineurin active site
Calcineurin phosphatase	Intact	Inhibited	Inhibited
Binding partner	None	Cyclophilin	FK506-binding protein (FKBP-12)
Side effects	Not observed	Nephrotoxicity, hypertension, neurotoxicity	Nephrotoxicity
Administration	Intraperitoneal, subcutaneous	Oral, intravenous (i.v.)	Topical, oral, i.v.
Potential application	Allograft transplantation, cardiac hypertrophy, restenosis	Transplantation, psoriasis, rheumatoid arthritis	Transplantation, eczema

Table 1. Physicochemical characteristics of VIVIT, CsA and FK506. Having similar clinical applications to CsA and FK506, VIVIT turns out to have no side effects, no toxicity and inhibits efficiently the binding of CN and NFAT. *Adapted from Yu et al 2007.*

As mentioned before, NFAT plays a critical role in T-cell mediated gene transcription regulation during inflammation and immune responses. Its activation will induce the expression of many cytokines, all of which were shown to contribute to CVDs such as atherosclerosis (Rao et al. 1997). To date, it is unclear whether inhibition of CN-NFAT signaling will prevent or ameliorate (transplant) atherosclerosis. Selective NFAT inhibition by VIVIT appears to be an effective strategy to coordinately quench inflammation and hypertrophy. Compared to CsA and FK506, VIVIT displays a favorable therapeutic profile, as it neither affects PKC-mediated extracellular signal nor CN-mediated NF-*κ*B activity, while being almost equally active in interdicting NFAT signaling. As a putative less toxic candidate than CsA or FK506, VIVIT will constitute not only a useful tool in exploring CN/NFAT signaling, but also lead the way to novel therapeutics for the treatment of cardiovascular disorders such as cardiac hypertrophy, restenosis, and atherosclerosis (Yu et al, 2007).

4.2.2.2. LxVP

Rodriguez and collaborators (2009) showed that a peptide based on the wild type LxVP sequence from NFAT inhibited both the phosphatase activity of CN toward a phosphopeptide in vitro, and the CN-dependent NFAT activation as well as NFAT-dependent transcription in cell cultures. Somewhat surprisingly, the same LxVP peptide stimulated, rather than inhibited, the phosphatase activity of CN toward a small-molecule, the para-nitrophenylphosphate (pNPP), reminiscent of the IS-IP complexes (Liu et al, 1991). Indeed, the LxVP peptide can compete with the two IS-IP complexes for CN binding, suggesting that the LxVP motif might interact with the same hydrophobic composite surface, formed by the two CN subunits. This idea was confirmed by analyzing the binding of CN proteins mutated within this composite site; those CN mutants that show diminished interaction with IS-IP complexes also suffered from a loss in binding to the LxVP peptide (Rodriguez et al, 2009). Since the CN hydrophobic docking surface is also essential for binding IS-IP (CsA and FK506) complexes, the authors propose that these drugs block the interaction of CN with LxVP-type motifs common to CN substrates, thereby inhibiting CN-dependent signalling.

As it was mentioned before, LxVP and PxIxIT both contribute to the overall affinity of the substrate for CN. The properties of LxVP, however, are distinct from those of PxIxIT. Peptides that contain either motif effectively inhibit the dephosphorylation of NFAT, but a peptide containing the LxVP sequence from NFAT specifically inhibits the interaction between NFAT and the activated form of CN. Mutations within the LxVP binding region in yeast CN broadly disrupt its function and interactions with substrates. These substrates include Rcn1, the yeast member of the conserved RCAN family of CN regulators, which contains a consensus LxVP site that is required for binding to CN. LxVP sites have been identified in additional mammalian substrates, such as RCAN and kinase suppressor of Ras 2 (KSR2) (Roy and Cyert, 2009).

5. GENE THERAPY IN CARDIOVASCULAR DISEASES

In spite of the remarkable therapeutic advances of the past 30 years, CVD remains the major cause of disability and premature death. Novel genetic tools are being developed to genetically modify the cardiovascular system and offer new possibilities for the treatment of CVDs. Gene transfer might be used as a gain-of-function strategy to silence or over-express genes that are involved in disease progression. The therapeutic potential of this new tool has been demonstrated in several animal models of CVD using a wide range of therapeutic targets. Despite these significant advances, there is still room for improvement in the vector engineering and its applicability in the clinical management of CVD (reviewed in Melo, 2005).

The current problems in gene therapy include lack tissue specificity and transient transgene expression, making them unsuitable for use in chronic CVD. Non-viral vectors have proven low yields and poor gene-transfer efficiency as they are non-integrative and are rapidly degraded. The most suitable vectors for gene transfer in cardiac tissues are recombinant viruses. These are replication-incompetent viral particles that deliver transgenes with high efficiency. Among these viral vectors, those derived from adeno-associated virus (AAV) or lentivirus, are capable of sustained expression of the therapeutic gene, rendering them suitable for use in chronic myocardial and vascular diseases. The main concern at the moment is focused on the

development of tissue-specific vectors able to direct the therapeutic transgene expression to a target tissue in response to pathophysiological stimuli, such as hypoxia and oxidative stress. Several studies are looking into novel approaches for local vascular gene delivery using stents coated with genetically engineered cells, plasmids or viral vectors expressing therapeutic genes (Melo, 2005).

As atherosclerosis is the most common CVD and inflammation participates pivotally in all stages of atherosclerosis, inflammation-regulated vector systems are promising tools for developing new gene transfer strategies for CVDs.

5.1. INFLAMMATION-REGULATED SYSTEMS

Chronic inflammatory diseases are characterized by relapse and remission episodes that often course with an acute type of inflammation superimposed on the inflammation already present. Ideally, the treatment of chronic inflammatory diseases should parallel the course of the proper disease itself, in order to match the variable pathological conditions and prevent undesirable secondary effects. An ideal vector system would be a disease-regulated one which expresses high levels of anti-inflammatory agents during relapses and low levels during remission phases of the disease.

Several viral expression systems which respond to inflammatory stimuli in vivo have been described so far (van de Loo et al, 2004). However, both the induction of mediated immune response and rapid loss of transgene expression due to the localization of the viral genomes hampered the therapeutic efficacy of these vectors. AAVs are promising candidates for long term gene therapy as they are not involved in any known human pathology, however, it has been recently shown that under some circumstances they may cause an inflammatory response in the host (Mingozzi and High, 2007; Zaiss et al, 2008; Peden et al, 2009).On the other hand, lentiviral vectors (LV) seem more appropriate to be employed in chronic inflammatory processes, as they can infect both dividing and quiescent cells, provide long-term expression, and display low immunogenicity (Buchschacher and Wong-Staal, 2000).

Currently, viral promoters are the most frequently used promoters for transgene expression, but these strong promoters are usually silenced, and result in only transient expression in vivo. Moreover, constant high levels of anti-inflammatory molecules might increase the risk of infection as already observed with anti-TNF-α and anti-IL-1 treatment of patients with rheumatoid arthritis (Roth et al, 2002; Mayordomo et al, 2002; Nuñez Martinez et al, 2001; Sicotte and Voskhul, 2001). In addition, there might be an adaptative response to the constant high concentrations of transgene protein, counteracting its therapeutic effect. Several vectors driven by drug-controlled promoters have been described to achieve regulated transgene expression. Still, their major drawback is the necessity for constant disease monitoring to achieve optimal efficacy, which is further complicated by the unpredictability and relapsing clinical course of the disease.

Objectives

There are two main objectives in this project:

I. Studying the role of Calcineurin in atherosclerosis by expressing CN-blocking peptides under the control of a lentiviral system in a murine model of experimental atherosclerosis.

II. Development of a new inflammation-regulated lentiviral system based on the E-selectin promoter.

Materials and Methods

1. Plasmid constructs

Human E-selectin promoter (-940;+40) (ESELp), was amplified by PCR from human genomic DNA. The PCR product was cloned into the pHRSIN HIV-derived transfer vector to replace the ubiquitously expressed Spleen Forming Focus Virus promoter, SFFVp. The firefly luciferase cDNA was amplified by PCR from a commercially available plasmid (pGL3 Basic). The PCR product was directly cloned into the SIN-BX plasmid to generate the bicistronic cassette. The luciferase-IRES-GFP was further cloned into the pHRSIN transfer plasmid by employing restriction endonucleases. The sequences of all plasmids were confirmed and are available at http://www.rodriguezlab.com and http://www.lablife.org/labs/947.

2. Lentivirus production and titration

HEK-293 cells were transiently transfected by the calcium phosphate method (Rodriguez & Flemington 1999). For the viral particles production, the indicated pHRSIN transfer plasmid was co-transfected with two helper plasmids (8.9 and pMD2-G). Supernatants from transiently transfected cells were collected 48h after transfection and cells debris removed by centrifugation (10min, 740xg, 4ºC). Viral particles concentration was obtained by ultracentrifugation in a swing bucket rotor for 2h at 121,896xg (RCF max), 4ºC (Ultraclear Tubes, SW28 rotor and Optima L-100 XP Ultracentrifuge, Beckman). After the spinning-down and removing the supernatant, viral particles were resuspended in 1xPBS and stored at -80ºC until used.

Total viral content was estimated /calculated by qPCR (Scherr et al 2001). Transducing Units (TU) were calculated by infecting 50,000 Jurkat cells in a p96 plate, with 1ul, 0,1ul and 0,01ul (duplicates) of concentrated viral supernatant. After 12h of infection, viruses were removed and after resuspending in 1xPBS, cells were analyzed by flow cytometry (FACS Canto HTS, Beckton Dickinson). The percentage of GFP positive cells was obtained and the number of infective particles/µl was calculated.

3. Cell culture and transient transfection

HEK-293 (ATCC #CRL-1573) cells were grown in Dulbecco's modified Eagle medium (DMEM, Bio-Whittaker, Lonza) supplemented with 10% of fetal bovine serum (FBS, Sigma) and L-glutamine plus antibiotics (100 units/ml penicillin and 100 µg/ml

streptomycin). Jurkat cells were cultured in RPMI medium (RPMI, Bio-Whittaker, Lonza) containing 10% FBS and L-glutamine plus antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). Primary endothelial cells (Human Umbilical Vein Endothelial Cells, HUVEC; Mouse Lung Endothelial Cells, MLEC) were isolated and grown in cell culture. HUVEC were serially passaged and cultured using 199 medium (Biowhittaker) supplemented with 20% FCS, bovine brain extract, and heparin in tissue culture flasks precoated with gelatin. Cells were used between passages 6 and 10 and were trypsinized before each pass. Primary MLEC were grown on a mixture of 10 mg/ml fibronectin (Sigma), and 0.1% gelatin (Sigma)-coated 75-cm flasks (Costar Corp.) with DMEM, Ham's F-12 (Gibco), 20% FBS, heparin (Sigma), glutamine (Sigma), and antibiotics. The cells were used for up to four passages. Immortalized MLEC (iMLEC) were grown in DMEM F-12 (Bio-Whittaker, Lonza) supplemented with 10% FBS, L-glutamine plus antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin), bovine brain extract and 50µg/ml ECGF (Hortelano et al. 2010). Cells were serum deprived for 12h prior to stimulation with the indicated growth factors and cytokines. After stimulation, cells were trypsinized and harvested for further analysis. Murine RAW macrophages (RAW 264.7 ATCC) were maintained in 10% FBS/DMEM F-12 and starved at 0.1% FBS when treated with LPS. After treatment, supernatant from the macrophages was collected and used to stimulate iMLEC, in order to mimic the effect of LPS injection in the in vivo model.

4. Flow cytometry, ELISA and luciferase measure

For analyzing GFP expression, cells were harvested, washed with 1xPBS and analyzed by flow cytometry (FACScanto, BD).

Cytokine levels present in serum were determined by commercially available kits following manufacturer recommendations (Quantikine Mouse IL-6 immune assay; R&D systems) and measured in a Benchmark Plus Microplate Spectrophotometer (BIORAD).

For analysis of luciferase activity, transduced cells were collected after stimulation, washed with 1xPBS, lysed, and luciferase activity determined in an AutoLumat LB953 luminometer (Berthold Technologies). Results are expressed in relative light units (RLU).

5. Animals and diets

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health.* For the atherosclerosis model, four weeks old male and female wild type and ApoE –KO mice (C57/BL6, Charles River), were maintained on a low-fat standard diet (2,8% fat, Panlab, Barcelona, Spain) and kept under a 12 h light/dark cycle. When indicated, ApoE-KO mice were fed a high fat cholesterol-rich diet (HFC-diet, 12% fat, 1,25% cholesterol, 0,5% sodium cholate, S8492-S010, Ssniff) and the CsA group were fed a high fat cholesterol-rich diet with a dose of 18 mg/kg/day of CsA (Calbiochem) incorporated in the diet (Ssniff). Blood was extracted through the retroorbital plexus and collected into EDTA tubes to obtain plasma. For the inflammation model C57/BL6 4-weeks-old male and female mice from Charles River were fed lab chow and kept under a 12 h light/dark cycle.

6. Quantification of atherosclerosis burden

Fat-fed mice were sacrificed and the organs were removed after in situ perfusion with PBS followed by 4% paraformaldehyde/PBS. Fixation was continued overnight. Aortas were cleaned from fat deposits around the tissue under the magnifying glass and kept in PBS at 4ºC. An operator who was blinded to genotype quantified the extent of atherosclerosis by computer-assisted morphometric analysis (SigmaScanPro5, Aspire Software International, Ashburn, Virginia) of both whole-mounted aorta stained with Oil Red O (0.2% Oil Red O in 80% MeOH) (Sigma) and hematoxylin/eosin-stained cross-sections of paraffin-embedded aortic root and ascending aorta as previously described (Gonzalez-Navarro et al, 2010).

7. Immunohistochemical analysis

7.1 Atherosclerotic plaques. Immunohistopathological examination of atheromas performed by a researcher blinded to genotype included the quantification of the content of macrophages, VSMCs, and collagen (Masson's trichrome stain) in ascending aorta and heart valves. VSMCs were identified with mouse anti-smooth muscle (SM) α-actin monoclonal alkaline phosphatase-conjugated antibody (1/20 dilution, clone 1A4) and Fast Red substrate (both from Sigma). Specificity was tested by substitution of primary antibody with non-related IgG. Macrophages were detected with a rat anti-

Mac3 monoclonal antibody (1/200 dilution, clone M3/84, sc-19991, Santa Cruz Biotechnology, Santa Cruz, California), followed by biotin-conjugated goat anti-rat secondary antibody (1/300 dilution, sc-2041, Santa Cruz Biotechnology), streptavidin-HRP (Ref. TS-060-HR, Lab Vision Corporation, Fremont, California), and DAB substrate (BUF021A, AbD Serotec). Specimens were counterstained with hematoxylin and analysis of the images was performed with MetaMorph v7.0 Meta Imaging Series (Molecular Devices Corporation, UK) (Gonzalez-Navarro et al, 2010).

7.2 Infectivity in Aorta and heart valves. Paraffin cross-sections of fixed ascending aorta and aortic root were obtained (5µm) and stained with anti-GFP antibody (Invitrogen, 1/100 dilution), to check infection efficiency in cardiac tissue.

***7.3 Kidney tissue*.** Paraffin cross-sections (5µm) from fixed kidneys from apoE mice were stained with hematoxylin/eosin to show tisular damage after each treatment and detect toxicity.

8. Immunoblot analysis

After stimulation, cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with lysis buffer (20 mM Hepes pH 7.6 containing 0.4 M NaCl, 1 mM EDTA, 3 mM EGTA, 1 µM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 100 µM benzamidine, 1 µg/ml of pepstatin, 1µg/ml of aprotinin, and 1% Triton X-100).

Lysates were incubated on a rocking platform for 15 min at 4°C and centrifuged at 14,000 x g (15 min, 4°C). Supernatants were then collected, 2X Laemmli buffer was added and extracts were boiled for 10 min. Proteins were separated under reducing conditions on SDS-polyacrylamide gels (6% for NFAT), and transferred to nitrocellulose membranes. Membranes were blocked with 5% skimmed milk (w/v) in TBS for 1 h at room temperature, washed several times with TBS-T (0.1% Tween-20 in PBS), and incubated overnight at 4°C in blocking buffer containing the appropriate primary antibody. Antibodies and dilutions were as follows: mouse monoclonal anti-NFATc1 (Alexis 7A6; 1/1,000), mouse monoclonal anti-NFATc2 (Abcam ab2722; 1/1,000).

After 3 x 5 min washes in TBS-T, blots were incubated for 1 h at room temperature with peroxidase labelled goat anti-mouse or anti-rabbit IgG (Pierce) (1/5,000 in blocking solution). After 3 x 5 min washes in TBS-T and one in water, membrane-bound

antibody was detected with enhanced chemiluminescence (ECL) detection reagent (Millipore).

9. In vivo optical imaging.

IVIS 200 (In vivo Imaging System; Caliper) was used for bioluminescent imaging analysis; mice were injected IP with 150 mg/kg firefly luciferin (Promega) 15 min before imaging and anesthetized with isoflurane during the procedure. Photons emitted from living mice were acquired as photons per s/cm2 per steradian by using Living Imaging 3.0 by Caliper. For photon quantification, a region of interest (ROI) was manually selected and kept constant within all experiments.

10. In vivo matrigel plug assay

Transduced iMLEC with lentiviral particles containing the luciferase cDNA were embedded in matrigel (SIGMA) which was subcutaneously injected in the mouse. Twenty four hours after matrigel implantation, lipopolyssaccharide (LPS from *E. coli*, 40mg/ml, SIGMA) was intraperitoneally injected and 4-6h later blood samples were obtained to determine the cytokine levels (IL-6) in serum by ELISA. Bioluminiscence was monitorized at 24h, 48h and 72h after LPS treatment using the IVIS 200 (In vivo Imaging System; Caliper) and images were analyzed with Living Imaging 3.0 (Caliper).

11. Zymosan Induced Arthritis (ZIA) animal model

Viral particles ($2x10^7$ TU) were injected in each paw of the mouse and saline solution (30ul) was injected in the contralateral paw as a negative control. One week after transduction, arthritis was induced in the right paw by intraarticular administration of 180□g of zymosan [23 Darren et al, 2009]. Luciferase expression was tracked by bioluminescence for one month every four days for the first two weeks and then every ten days, using the IVIS 200 (In vivo Imaging System; Caliper). Inflammation was determined by paw diameter measure and luminol assay (Sigma-Aldrich).

12. Reverse Transcription and Real Time PCR Analysis

Total RNA from Jurkat cells was extracted with TRIZOL (Sigma) and 2μg was reverse transcribed to cDNA with MMLV-RT (Invitrogen). Real-time PCR was carried out using TaqMan Low Density Arrays (Immune panel; Applied Biosystems) following the manufacturer's recommendations. PCR reactions were run in triplicate in an ABI Prism 7900 HT-Fast Real-Time PCR System (Applied Biosystems).

Total RNA from aortas was extracted by employing RNeasy Fibrous Tissue Mini Kit (Qiagen) and reverse transcribed with the RT2 PCR Array First Strand Kit (Qiagen). Real-time PCR was carried out using Atherosclerosis PCR Arrays (Mouse Atherosclerosis RT² Profiler) following the manufacturer's protocol (Qiagen). PCR reactions were run in triplicate in a Step One Plus Real-Time PCR system (Applied Biosystems).

13. Statistical analysis

Statistical analysis was performed by employing the GraphPad Prism software (version 5.01). Data shown in figures 15 and 24 were analyzed by one-way ANOVA followed by Newman-Keuls multiple comparison test. Data shown in scatter plots were analyzed by a paired t test (Figures 23-24). Statistical significance was assigned at $p < 0.05$.

Results

PART I. INHIBITION OF CN PATHWAY BY EXPRESSING CN-BLOCKING PEPTIDES IN A LENTIVIRAL SYSTEM: ROLE OF CN IN ATHEROSCLEROSIS.

The first part of this study consisted in proving the therapeutic effect of two CN blocking peptides, VIVIT and LxVP, in a murine model of experimental atherosclerosis

1.1 Lentiviral vectors carrying calcineurin blocking peptides impair the NFAT signaling in cell cultures.

For the delivery and expression of these peptides we have employed a lentiviral vector (LV) system which expresses the transgene (Green Fluorescent Protein [GFP]=control; VIVIT-GFP=VIVIT; GFP-LxVPc1=LXVP) under the control of a strong promoter from Spleen Forming Focus Virus (SFFVp) (Figure 10A). These LVs were tested in vitro in iMLEC in order to check the blockage of the CN/NFAT pathway. Our results show that only the peptide-expressing LVs (VIVIT and LxVP) efficiently block the NFAT dephosphorylation by active CN upon intracellular calcium increase in EC (e.g. ionophore treatment) (Figure 10B). In contrast, the control vector (LV-GFP) did not affect the NFAT dephosphorylation. In addition to the NFAT phosphorylation state, we examined the impact of these peptides in the transcriptional regulation of known NFAT dependent genes of the cytokine network. Our results showed that infection with LV-LxVP and LV-VIVIT, but not LV-GFP, severely reduced the inducible mRNA expression of IL-2, IL-3, IL-8 and TNF-α (Table 2). These results are in agreement with those already published (Aramburu et al, 1999; Rodriguez et al, 2009).

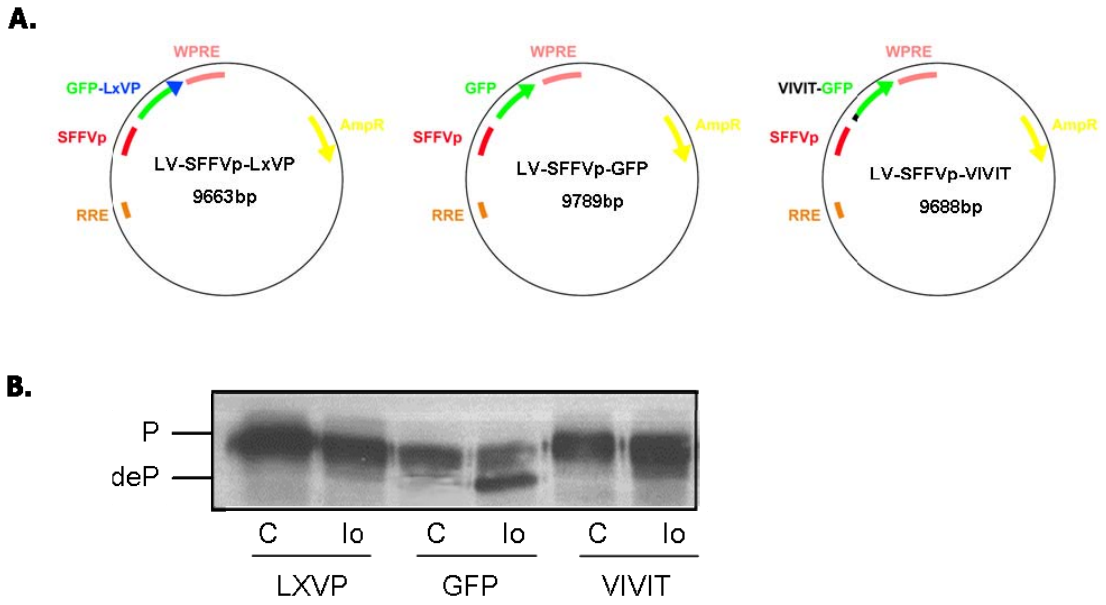

Figure 10. Lentiviral vectors containing the CN-blocking peptides inhibit NFAT dephosphorylation. (A) Plasmid maps of the transfer vectors employed. LXVP (left) and VIVIT (right) peptides were expressed fused to GFP under the strong viral promoter SFFVp. The parental transfer vector (center) carrying GFP was used as a control. **(B)** NFAT dephosphorylation analysis. iMLEC infected with the indicated LV were treated (Io) or not (C) with calcium ionophore which activates calcineurin. Cell extracts were analyzed by western-blotting by employing anti-NFATc1 antibody. P=phosphorylated; deP=dephosphorylated.

To study the effect of the blocking peptides in the NFAT-dependent transcriptional regulation we measured the RNA levels of NFAT-target genes. Jurkat cells transduced with the indicated LVs were stimulated (phorbol ester plus Io) for 4 h, and total RNA was extracted and analyzed in a low density gene expression array. As shown in table 2, expression of the NFAT-dependent cytokines IL-2, IL-3, IL-8 and TNFa, normally induced during lymphocyte activation, was efficiently downregulated by the expression of CN-blocking.

	LV-GFP		LV-LXVP		LV-VIVIT	
GENE	control	PMA+Io	control	PMA+Io	control	PMA+Io
IL-2	1.0±0.0	485.6±37.1	0.8±0.1	123.1±30.2	0.5±0.0	21.8± 3.4
IL-3	1.0±0.0	580.3±44.4	0.4±0.0	89.4±21.9	0.3±0.0	3.6± 0.6
IL-8	1.0±0.0	155.5±11.9	1.1±0.1	47.9±11.7	0.8±0.1	71.4±11.1
TNF-α	1.0±0.0	12.5± 0.9	0.9±0.1	7.9± 1.9	1.3±0.1	1.4± 0.2

Table 2. Expression of CN-blocking peptides downregulates the inducible transcription of NFAT-dependent cytokines. Jurkat cells transduced with the indicated LVs were stimulated with phorbol ester (PMA) plus ionophore for 4 h. Total RNA was extracted and analyzed by gene expression array. Values correspond to the fold induction (mean ± SD) relative to the expression in non-stimulated LV-GFP control cells. Three endogenous control (housekeeping) genes were employed.

1.2 Lentiviral vectors efficiently transduce blood vessel walls

Regarding all the advantages that the apoE model meets, we found this model suitable for our study, given its accelerated development of the disease on a high cholesterol diet, as well as its similarity to the development of the disease in humans.

To test if the LVs were efficiently delivered to the vessel wall we administered them intravenously (intra-jugular) into apoE animals. One week after the infection, the animals were put on a high-fat diet for two months (experimental strategy depicted in figure 11). In addition to these animal groups, we have included another group of animals fed with a CsA-containing high-fat chow (de Frutos et al, 2009). In these animals the CN signalling pathway is disrupted by the action of CsA, allowing us to compare the pharmacological inhibition of CN with the effect obtained by the expression of the CN blocking peptides.

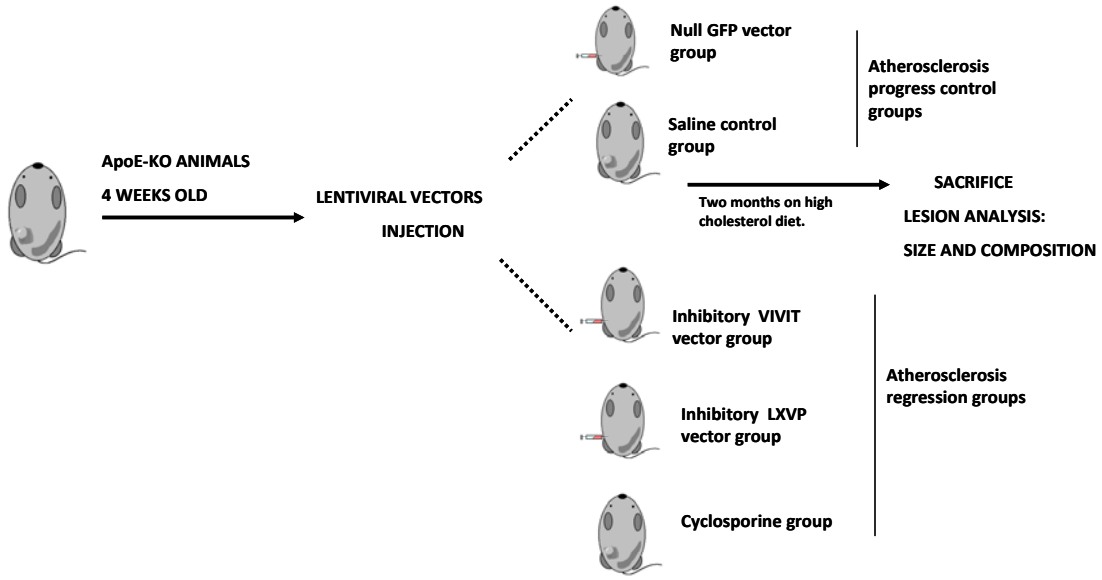

Figure 11. Schematic diagram showing the experimental strategy. The scheme shows the experimental strategy from the virus injection to the organ harvest and tissue analysis. The animals were fed with high-cholesterol diet during the whole experiment.

After two months on a high fat-diet, cross-sections of ascending aorta and aortic root (aka heart valves) were stained with anti-GFP antibody to identify the transgene expressing cells. As shown in figure 12, GFP positive ECs and VSMCs were detected only in LV-infected animals. GFP positive cells were detected both in aorta and in heart valves. As expected, no GFP positive cells were detected in cross-sections from control animals (i.e., PBS injected or CsA treated).

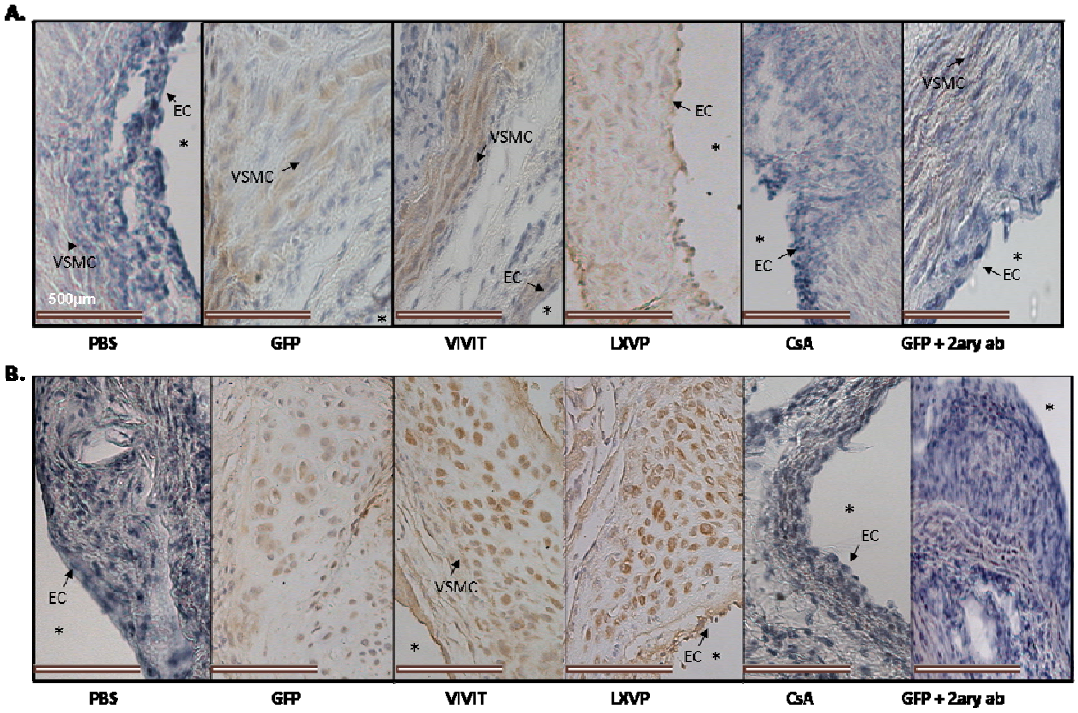

Figure 12. GFP expression in cardiac tissues. GFP staining obtained in cross-sections of **(A)** ascending aorta and **(B)** aortic root. GFP-positive (in brown) EC and VSMC were detected only in the GFP-expressing groups (GFP, VIVIT, LXVP); negative staining was obtained in control groups (PBS and CsA) which was comparable to that obtained in the presence of only the secondary peroxidase-labeled antibody. Asterisk indicates the lumen. The layers of EC and VSMC are indicated with arrows.

In order to confirm the transcriptional inhibition of the CN-NFAT pathway, we examined the RNA level of NFAT-dependent genes in the aorta of LV-infected animals. Our results showed that the expression of the CN-blocking peptides, LxVP or VIVIT, reduced the transcriptional expression of known NFAT-regulated genes such as TNF-α and IFN-γ (figure 13A). These results confirm that the CN/NFAT pathway is efficiently hampered by the CN-blocking peptide expression in the aorta.

To monitor the systemic CsA treatment effectiveness, lung cell extracts were made from CsA-treated animals and the NFAT phosphorylation state was studied by western-blotting. In all the CsA-treated samples analyzed, NFAT proteins were highly phosphorylated (Figure 13B); the NFAT phosphorylation pattern under CsA treatment was similar to that found in samples from animals on a normal diet. When samples from animals on a high-fat diet and on a normal diet were compared, we found that high-fat diet is able to induce NFAT dephosphorylation which can be at least partially blocked by CsA treatment.

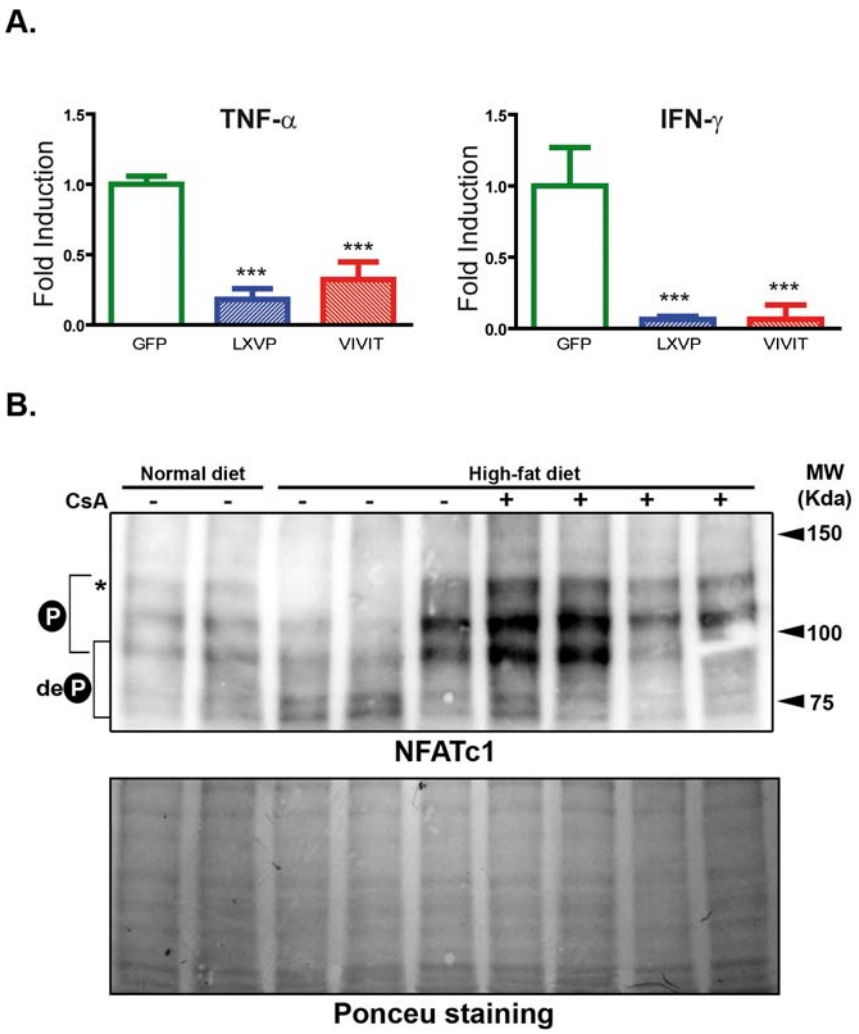

Figure 13. CsA treatment and CN-blocking peptides inhibit the CN/NFAT pathway. **(A)** CN-blocking peptide expression blocks the transcription of NFAT-dependent genes. RNA levels of the NFAT-dependent genes, TNF-α and IFN-γ, from the aorta of LV-infected animals were analyzed by qPCR. The expression of LXVP or VIVIT efficiently blocked the transcription of these two cytokines. ($n \geq 5$; ***$p<0.0001$ vs GFP) **(B)** CsA administration blocks NFAT dephosphorylation. Lung extracts from animals on a normal diet or on a high fat diet were analyzed by anti-NFATc1 western-blotting (top). The NFAT phosphorylation pattern under CsA treatment (top pannel) was similar to that found in samples from animals on a normal diet. High-fat diet is able to induce NFAT dephosphorylation which can be at least partially blocked by CsA treatment. Ponceau staining was performed to check uniformity in the quantity of protein per well (bottom). Asterisk indicates hyperphosphorylated NFAT. P=phosphorylated; deP=dephosphorylated.

1.3 Inhibition of calcineurin activity attenuates the atheroma burden in the aorta of apoE mice.

After virus injection and two months on a high fat diet, animal euthanasia was performed and target organs were extracted for further analysis. The atherosclerotic burden was measured in aorta and heart valves. We quantified the atherosclerosis burden by planimetric analysis of whole-mounted oil Red-O-stained aortic arch and

thoracic aorta (Figure 14). Due to the different blood flow conditions and morphogical characteristics, the atherosclerosis progress is different in the aortic arch and the thoracic aorta; for this reason the lesion quantification and analysis are done separately.

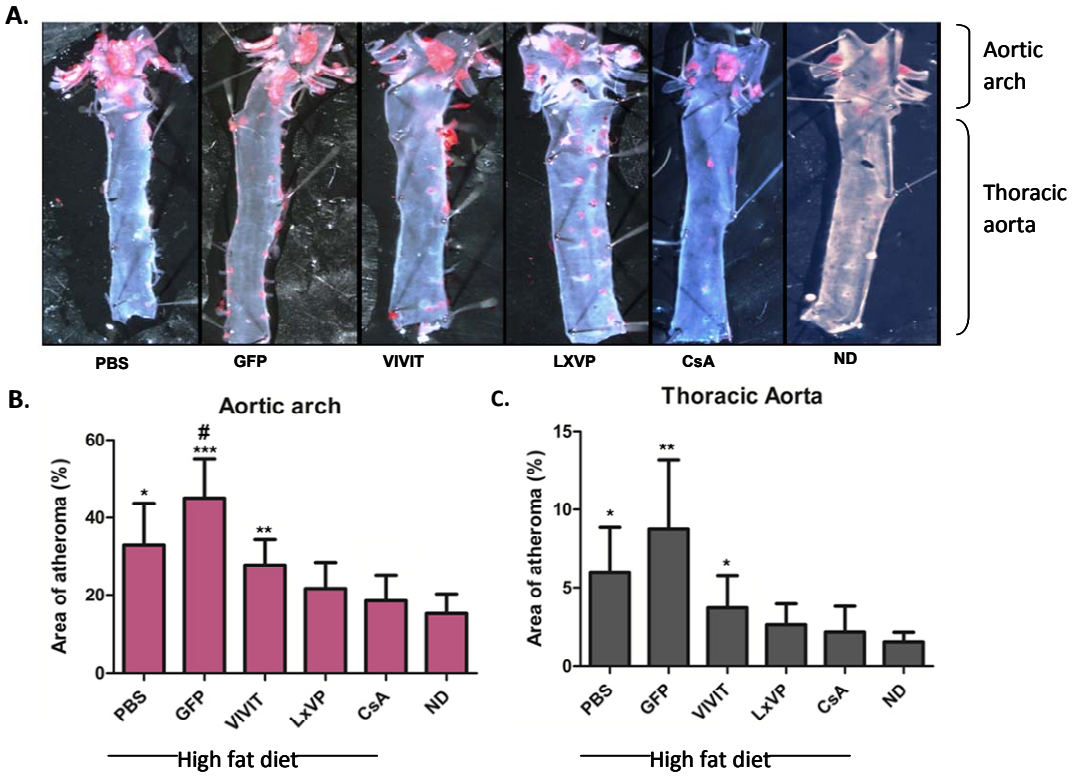

Figure 14. Inhibition of CN activity lowers the atherosclerosis burden in the aorta. (A) Aortas from apoE mice were extracted and stained with Oil-Red. Quantification of lesion area in **(B)** aortic arch and **(C)** thoracic aorta. Normal diet group (ND) was employed as a control group. * p<0.05 versus ND; # p<0.05 versus PBS. N= 12 animals per group.

When comparing the groups fed on high fat diet with the normal diet (ND) group we found that only the LxVP peptide-carrying group and the CsA-treated group lowered the high-fat induced atherosclerosis burden to normal diet levels in both aortic arch and thoracic aortha (Figure 14B-C).

In the aortic arch, when the GFP and VIVIT groups were compared to the PBS control group, we obtained significant difference only in the case of the GFP group (# p<0.05). This difference in the atheroma burden in aortic arch between the PBS and the GFP groups groups suggests that the infection by itself somehow accelerates the progress of the plaque. The LV infection may contribute to the atherosclerois by either

increasing the inflammation and/or affecting the endothelium making it more vulnerable.

When the VIVIT group was compared to the GFP control group, we found that the attenuation in the atheroma burden was statistically significant ($p<0.0001$ in aortic arch, and $p<0.001$ in thoracic aorta). Therefore, this analysis shows that the atheroma burden induced by the LV infection was inhibited by the VIVIT peptide, suggesting that the activation of the CN/NFAT pathway is required for the atherosclerosis rise upon LV infection. As the thoracic aorta is a less athero-susceptible area than the aortic arch (Davies et al, 2010), the differences found are less dramatic but the results were very similar: CsA-treatment and LXVP expression lowered the high-fat induced atherosclerosis burden to normal diet levels and the VIVIT expression was able to block atherosclerosis induced by the LV-infection.

We next quantified atherosclerotic burden in the heart by analyzing cross-sections through the aortic root and ascending aorta. These two areas in the aorta, along with the aortic sinus (an enlarged area found after the aortic valve) are very vulnerable places in the heart where the formation atherosclerotic plaque usually initiates. Therefore, these are the areas used to quantify atherosclerosis burden. Cross-sections were stained with haematoxilyn/eosin (H/E) to localize and quantify the lesion (Figure 15). Atherosclerosis burden was determined as lesion area in cross sections of aortic root and ascending aorta. Although no statistically significant differences were obtained in any of the regions when VIVIT and LXVP samples were compared to those from PBS and GFP control groups ($p>0.5$), the trend shows lower values in the case of VIVIT and LXVP groups.

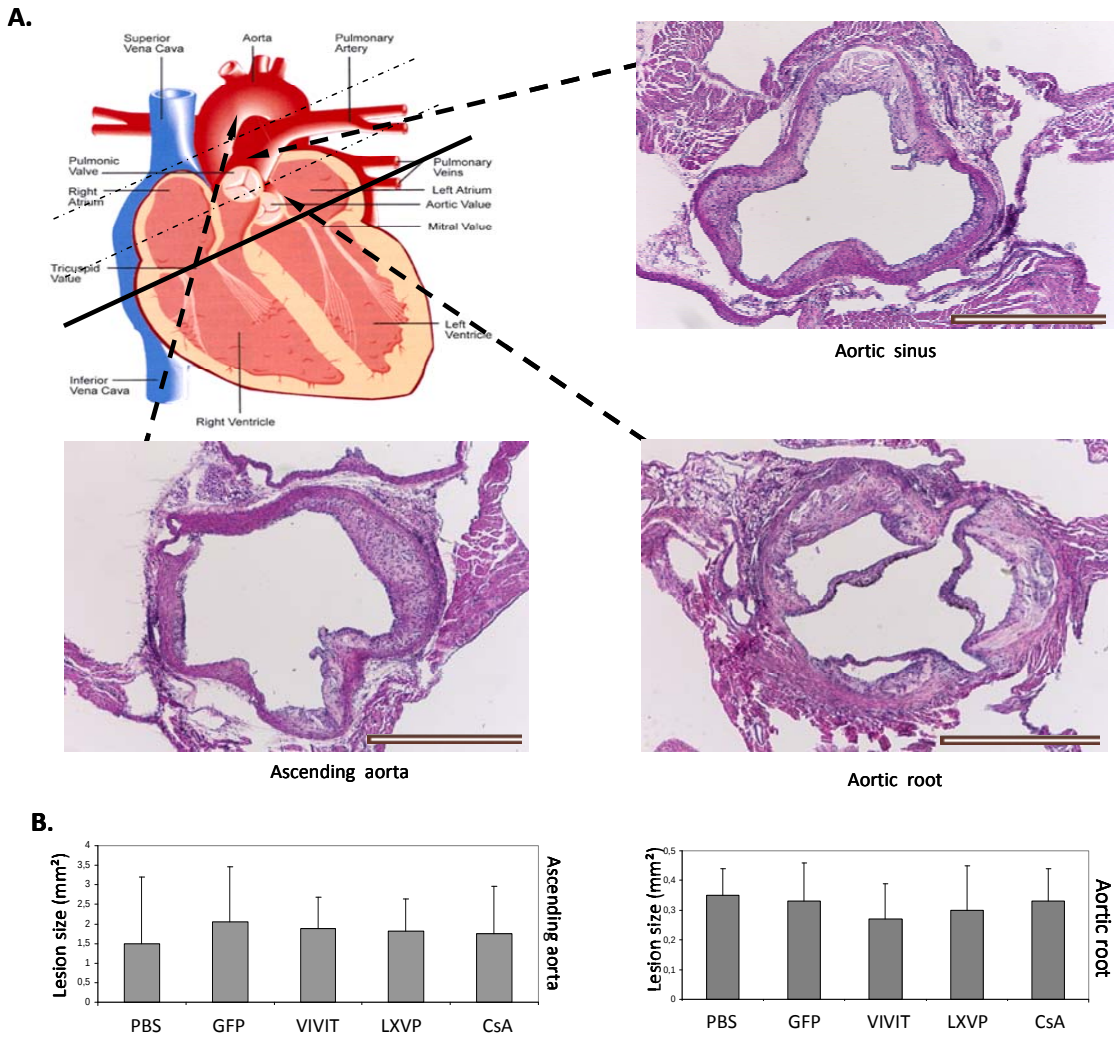

Figure 15. Lesion quantification of the heart. (A) Schematic diagram of the heart and aortic root. The cross lines in the heart diagram show the three main areas where the atherosclerotic burden was measured: aortic root, aortic sinus and ascending aorta. Cross-sections in haematoxilyn/eosin staining of these three areas enable to identify and quantify the lesion area**.** The bar is equivalent to 500µm **(B)** Atherosclerosis burden determined as lesional area (square milimeters) in cross sections of the aortic root and ascending aorta (for each mouse, average of three cross-sections separated by approximately 9µm). For statistical analysis Student t test was used. No significant difference was observed. p>0.5 versus GFP or PBS.

1.4 CN blockade does not affect to the plaque composition.

Lesion composition of the apoE mice plaques is similar to that found in human plaques. They progress from foam cell stage to the fibroproliferative stage with visible fibrous caps and lipid necrotic cores. After 8 weeks on a high cholesterol diet, we expect lesions rich in fatty streaks comprised of foam cells and migrating VSMCs. In areas such as the heart valves, lesions may have progressed to a more advanced phase with a heterogeneous necrotic core with visible cholesterol crystals (Jaiwen et al, 2004). Cross-sections from the aortic root and the ascending aorta were stained with

different antibodies to evaluate the composition of the plaque and quantify it. The content of macrophages was determined with anti Mac-3 (Gonzalez-Navarro et al, 2010); Mac-3 is a protein expressed on the surface of mouse mononuclear phagocytes, and its expression increases during differentiation of monocytes to activated peritoneal macrophages (Flotte et al, 1983).

We also quantified the migration VSMCs to the lesion with the alfa-actin antibody that stains smooth muscle cells in endothelium and other tissues. Additionally, the Masson tricromic staining is suited for distinguishing cells from surrounding connective tissue (red for muscle fiber, green or blue for collagen and pink for the cytoplasm of the cells). The amount of collagen in the lesion indicates the severity of the lesion and its stage. There were no significant differences ($p>0.5$) in the plaque composition among the different experimental groups in either of the sites selected for determine lesion composition, ascending aorta and aortic root (Figure 16).

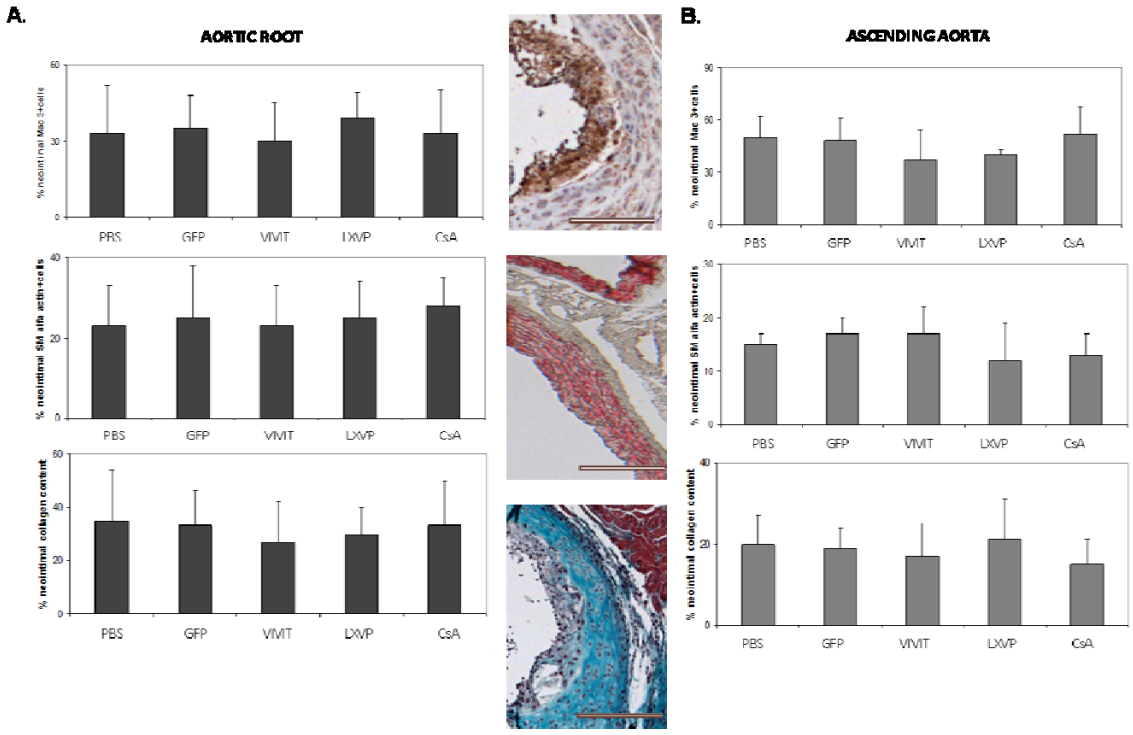

Figure 16. Plaque composition is not affected by blocking the CN pathway. Cross-sections from aortic root **(A)** and ascending aorta **(B)** were stained with anti-Mac3, anti-SM α-actin, and Masson's trichrome to quantify the neointimal content of macrophages, vascular smooth muscle cells (VSMCs), and collagen, respectively. Quantification is shown as percentage of stained area relative to the area occupied by atheroma. The photomicrographs (measure bar corresponds to 500µm) show

representative images of aortic root sections. Differences between groups were evaluated by the Student *t* test.

1.5 CsA treatment, but not the LV infection, caused nephrotoxicity.

It is well known that CsA treatment is associated with severe side effects, being the nephrotoxicity the major damage described in animal models. Therefore, we decided to examine cross-sections from kidneys of CsA-treated animals and compared them to those obtained from either control or LV-injected animals. We examined samples from 3 different animals per group and only found damage (hemorrhage, vasoconstriction or necrosis) in those obtained from the CsA-treated group (Figure 17). We have shown that the beneficial effects in terms of atherosclerosis obtained by CsA treatment and the expression of CN blocking peptides are comparable; therefore, as LV infection did not damage the kidneys, our results suggest that the blockade of CN signaling by employing these peptides attenuates the atheroma burden with, at least, no renal failure associated.

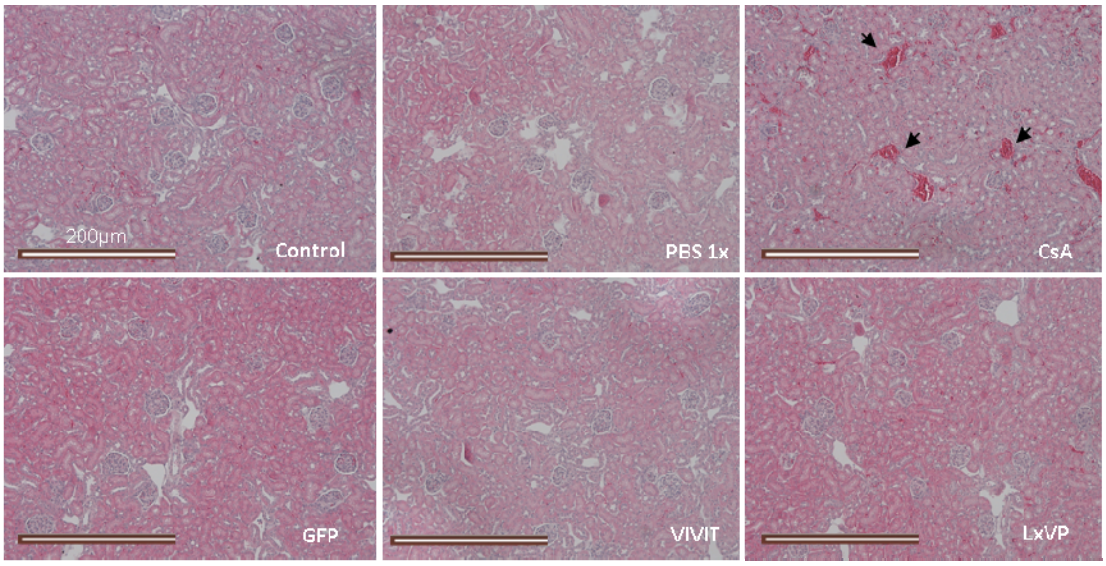

Figure 17. CsA treatment causes nephrotoxixity. Kidney cross-sections were stained (H/E) and the renal structural integrity was evaluated. Intratisular hemorrhages (arrows) were detected only in samples from CsA-treated animals. Bars correspond to 200µm.

PART II. DEVELOPING AN INFLAMMATION-REGULATED LENTIVIRAL VECTOR BASED ON THE E-SELECTIN PROMOTER FOR LONG TERM TREATMENT OF CHRONIC INFLAMMATORY DISEASES.

Inflammation is now considered a constant and key process present in all stages of the atherosclerotic disease, from lesion initiation to progression and destabilization. In fact, atherosclerosis is associated with chronic inflammation (Smolen et al, 2003). As we successfully employed LV for transgene expression in the cardiovascular system in vivo, we decided to develop an LV expression system able to parallel the course of a chronic inflammatory disease. In our research project, we decided to generate an inflammation-regulated LV expression system based on the E-selectin promoter (ESELp) and test it *in vitro* and *in vivo*.

2.1 Lentiviral vectors efficiently transduce human and murine endothelial cells.

To assess the ability of LVs to efficiently transduce endothelial cells, we infected primary endothelial cell cultures from murine (MLEC) and human (HUVEC) origin with a LV encoding GFP under the control of the SFFVp (LV-SFFVp-GFP). In addition, we infected immortalized MLEC (iMLEC) and analyzed GFP expression after 48h. The efficiency of transduction observed was close to 100% in all cases (Figure 18).

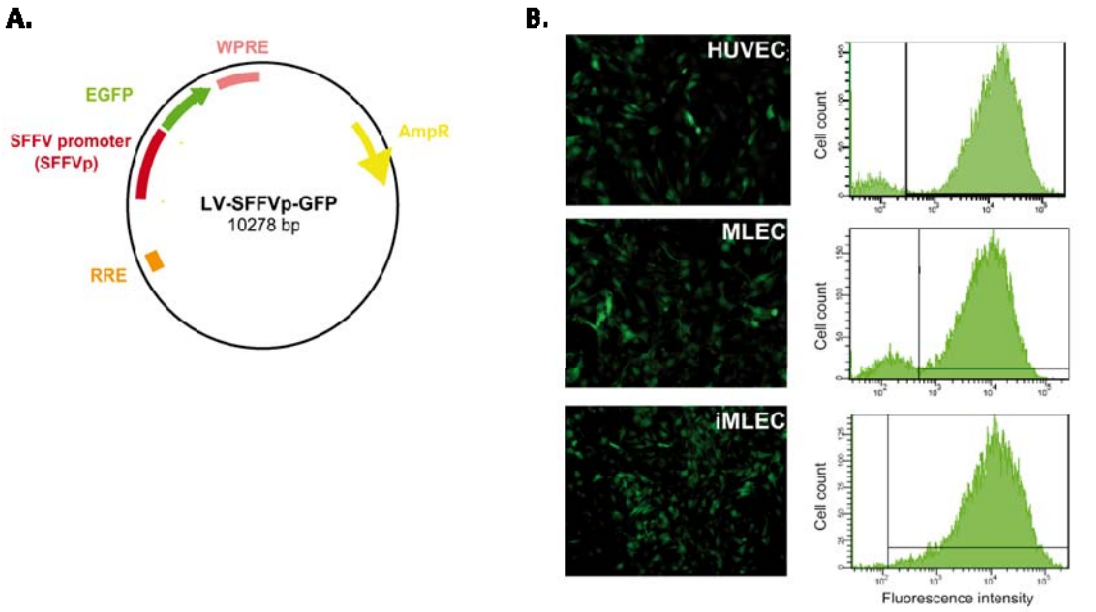

Figure 18. Endothelial cells are efficiently infected by lentivectors in vitro. (A) Plasmid map of the transfer lentivector employed which expresses GFP under the control of a strong viral promoter, SFFVp (cPPT=central PolyPurine Track; RRE=Rev-Responsive Element ; GFP=Green Fluorescence Protein; IRES=Internal Ribosome Entry Sequence; SFFV=Spleen Focus Forming Virus; WPRE=Woodchuck Post-transcriptional Element;). **(B)** HUVEC (top), MLEC (middle) and immortalized MLEC (bottom) were infected with 1200 PFU of LV-SFFVp-GFP, and after 48h GFP expression was assessed by fluorescence microscopy (left panels) and flow cytometry (right panels).

2.2 ESELp-driven transgene expression is efficiently activated by proinflammatory cytokines in lentiviral transduced endothelial cells

As E-selectin is the earliest endothelial specific adhesion molecule induced by pro-inflammatory cytokines, we tested whether the E-selectin promoter (ESELp) could be a useful tool to specifically direct transgene expression at sites of inflammation. We generated a lentivector encoding GFP controlled by ESELp (LV-ESELp-GFP; Figure 19) and infected both iMLEC and HUVEC.

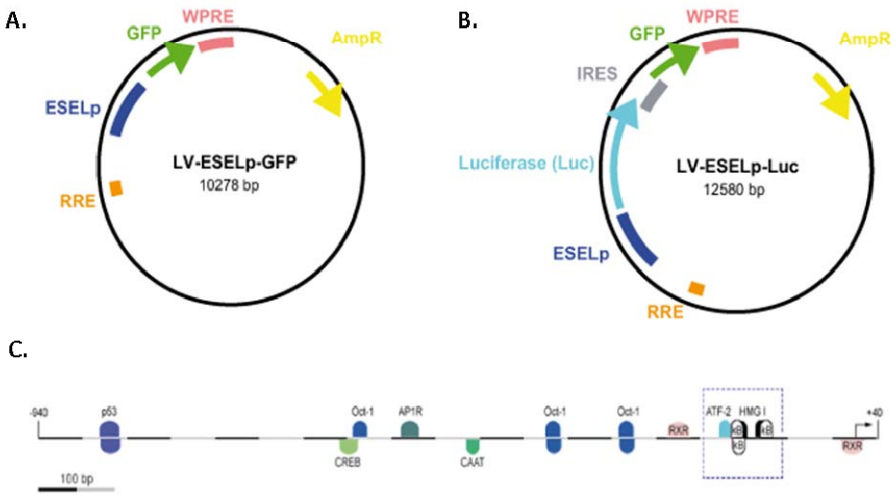

Figure 19. Lentivectors containing the ESEL promoter sequence. Plasmid map of the HIV-derived lentiviral vector which contains **(A)** the GFP transgene (LV-ESELp-GFP) or **(B)** the Luciferase-IRES-GFP bicistronic cassette under the control of the ESELp (LV-ESELp-Luc). **(C)** Organization of the regulatory elements found in the E-selectin promoter. Predicted transcription factor binding sites identified by employing the MatInspector software and the Matrix Family Library (version 8.2) from Genomatix (www.genomatix.de). The proximal region (first 160 base pairs) which has been fully characterized is indicated (box).

Treatment of infected cells with TNF-α resulted in a strong increase of GFP expression in both cell types; this increase paralleled the expression of endogenous E-selectin (Figure 20 A-B). In contrast, the GFP expression from the constitutively active LV-SFFVp-GFP was not modified by TNF-α treatment. Although TNF-α is a potent inducer of endogenous ESEL *in vitro*, preincubation of endothelial cells with the proangiogenic factor VEGF greatly enhances the induction achieved by TNF-α alone (Stannard et al, 2007). For this reason, we preincubated the infected cells for 24 h with VEGF and then with TNF-α for different time periods. The induction of GFP expression peaked at 6h both in VEGF and vehicle pretreated cells, and declined after 12h; as in the case of endogenous E-selectin, VEGF cooperated with TNF-α (Figure 20C).

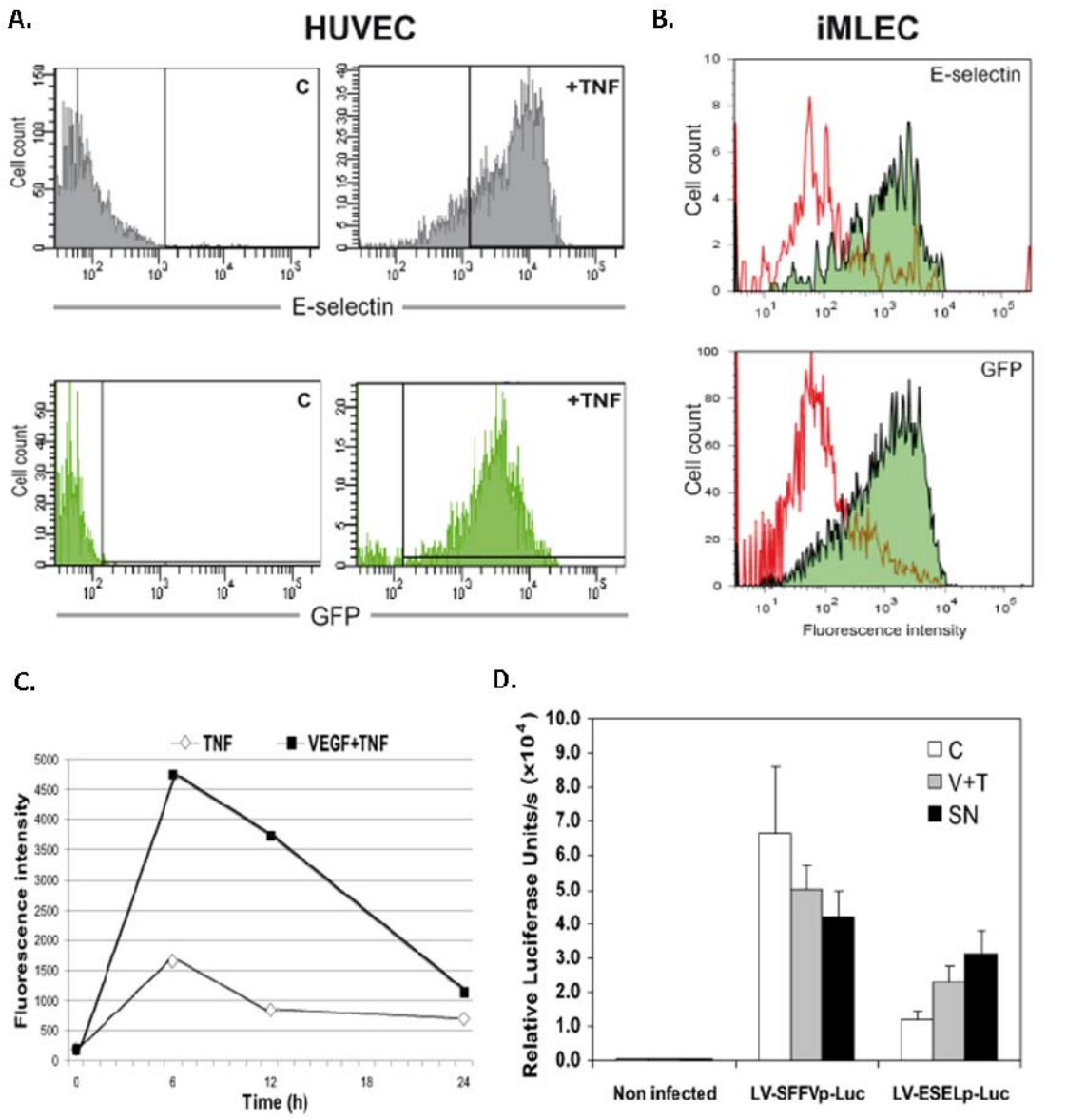

Figure 20. Inducibility of the ESELp-based lentiviral system in vitro. (A) HUVEC were infected with a lentivector encoding GFP driven by the ESEL promoter (LV-ESELp-GFP), and left untreated (left) or incubated with 30ng/ml TNF-α (right) for 6h. Flow cytometry histograms show the expression of endogenous E-selectin (top panels) and GFP (bottom panels) in control and TNF-a treated LV-ESELp-GFP transduced cells. **(B)** Infected iMLEC with LV-ESELp-GFP were treated as in (a). Histogram plots show the fluorescence intensity of untreated (gray line) or TNF-treated (filled) cells. **(C)** HUVEC were infected with LV-ESELp-GFP and either left untreated or preincubated with 50ng/ml VEGF for 24h. Cells were then incubated with 30ng/ml TNF-α for 6, 12 and 24h, and GFP expression analyzed by flow cytometry. Graph shows mean GFP fluorescence intensity in each condition from a representative experiment out of three performed. **(D)** iMLEC were infected with the indicated LUC-containing lentivectors. Twenty four hours after infection, cells were treated with VEGF plus TNFα (grey bars) or with the supernatant obtained from the LPS-treated RAW cells (black bars). After 30h, luciferase activity was measured in a luminometer. Graph shows relative light units (RLU) in each condition.

We next investigated whether the ESELp-based LV could be activated in an inflammatory context *in vivo*. The challenge of RAW 264.7, a murine macrophage-like cell line, for 4-6h with lipopolysaccharide (LPS), a component of the outer membrane

of gram-negative bacteria, stimulates the production in these cells of pro-inflamatory mediators such as TNF-α, IL-6, IL-1β, nitric oxide or cyclo-oxygenase-2, that are released to the cell media (Khan et al, 2011; Park et al, 2011). This cytokine cocktail can be used as pro-inflammatory stimuli simulating an *in vivo* inflammatory reaction to activate endothelial cells *in vitro.*

Therefore, we first tested the luciferase activity of cells transduced with a LV containing this reporter under the ESELp (LV-ESELp-Luc; Figure 19) in response to culture supernatant containing inflammatory mediators from LPS-activated macrophages (Giron et al, 2008). iMLEC transduced with LV-ESELp-Luc displayed the strongest luciferase activity in response to the cytokine-containing supernatant, whereas no induction was found in the case of LV-SFFVp-Luc transduced cells (Figure 20). The treatment with LPS alone did not modify either SFFVp-driven or ESELp-driven luciferase activity (data not shown).

2.3 ESELp-driven transgene expression is induced by proinflammatory cytokines in a murine model of subcutaneous matrigel

In animal studies, the administration of LPS triggers the host innate immunity by stimulating production of pro-inflammatory mediators and induces the transcription of cytokines in the spleen as well as in other organs, such as the liver. During infection, LPS is released in the circulation, where it stimulates the toll-like receptor-4 (TLR4)-MD2-CD14 receptor complex on inflammatory cells, which release massive amounts of pro-inflammatory mediators such as TNF-α, nitric oxide (NO), and arachidonic acid (Lin et al, 2011). On the molecular level it induces the synthesis of potentially cytotoxic factors (NO, IL-1, TNF-α, prostaglandins), the cell-surface expression of glycoproteins involved in cell adhesion (ICAM-1, P- and E-selectin), and many granulocyte-chemoattractive molecules such as IL-8 (Bohatschek et al, 2001). Therefore, we tested the inducibility of LV-ESELp-Luc in vivo after LPS administration. To this end, we performed subcutaneous matrigel experiments in mice to prove the LV-ESELp-Luc induction in the presence of LPS-induced pro-inflammatory mediators. A detailed scheme of this model is presented below (Figure 21):

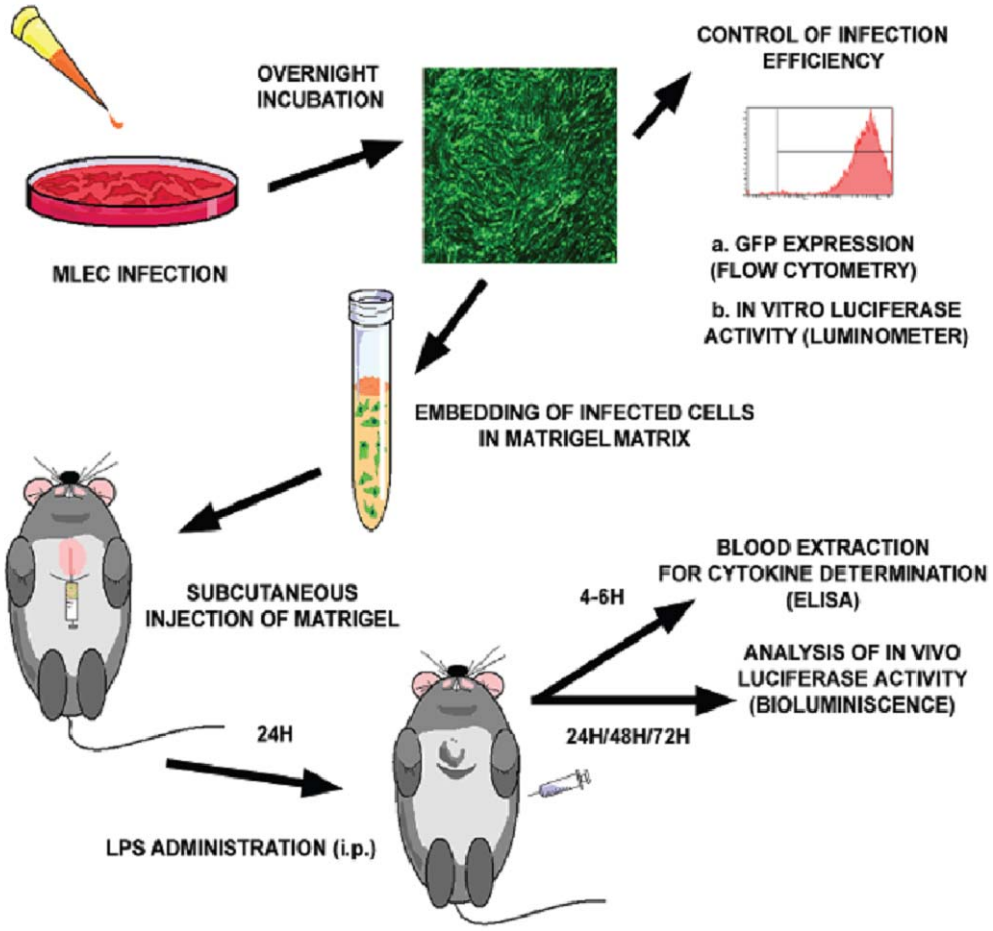

Figure 21. ESELp-driven transgene expression is induced by proinflammatory cytokines in a murine model of subcutaneous matrigel. MLEC cells were transduced with LV-SFFVp-Luc or LV-ESELp-Luc constructs. Infection efficiency was determined by GFP expression by flow cytometry and luciferase activity by luminometer. After 24h, cells were embedded in VEGF-containing matrigel and injected subcutaneously in syngenic mice. Twenty four hours later, mice were treated with LPS (i.p.), and luciferase activity in matrigel plugs was monitored by bioluminescence and 4 to 6h after the LPS injection blood was extracted to determine cytokine levels by ELISA. *Courtesy of Dr Alfranca*.

In these experiments, we transduced MLEC with LV-SFFVp-Luc or LV-ESELp-Luc constructs. After 24h, cells were embedded in VEGF-containing matrigel and injected subcutaneously in syngenic mice. Twenty four hours later, mice were treated with LPS (i.p.), and luciferase activity in matrigel plugs was monitored by bioluminescence.

Although LPS administration led to a similar increase in serum IL-6 levels of both LV-SFFVp-Luc and LV-ESELp-Luc mice, we found an increase in luciferase activity only in LV-ESELp-Luc, thus confirming the selectivity of ESELp induction by inflammatory cytokines in vivo (Figure 22).

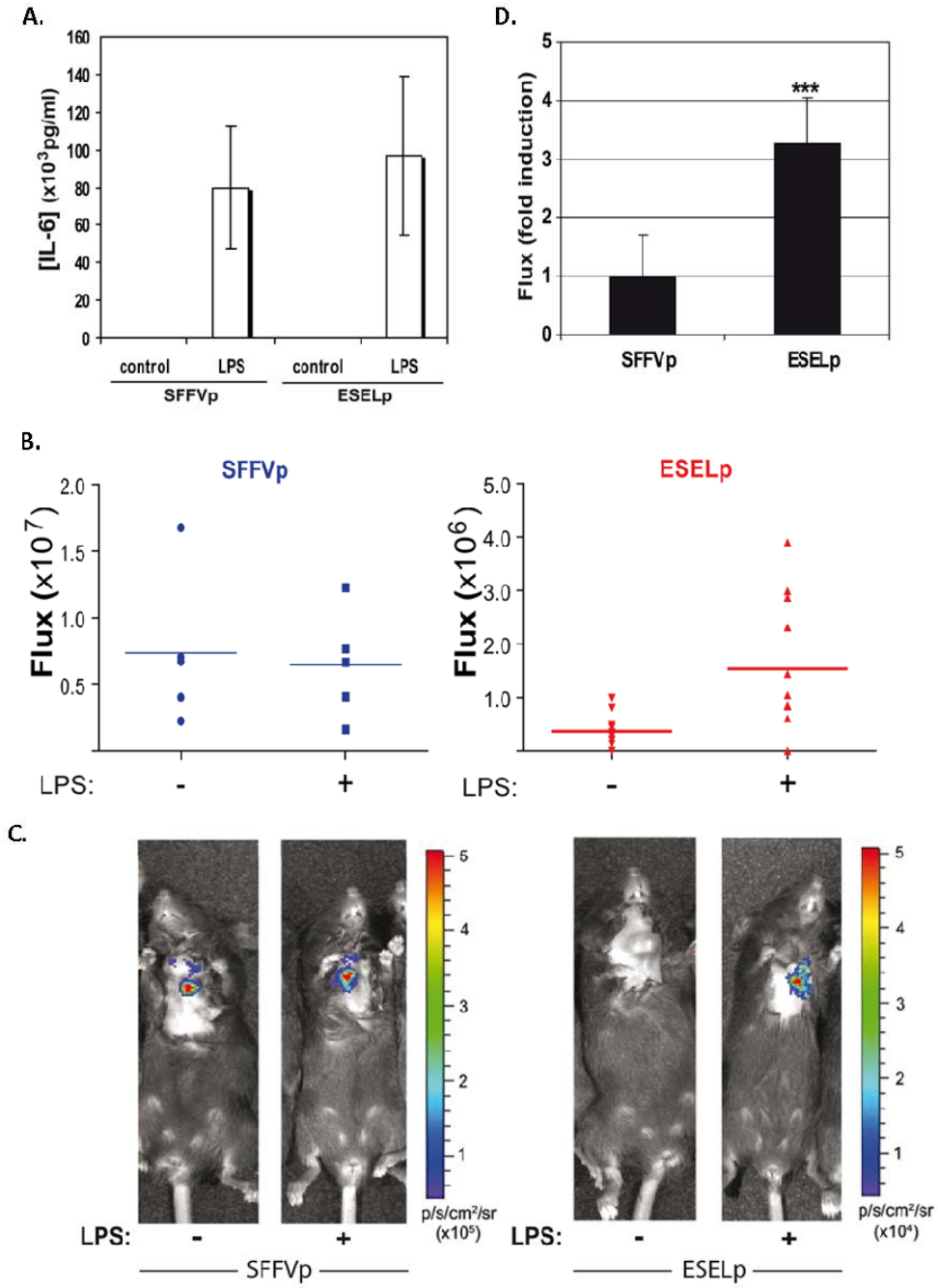

Figure 22. In vivo induction of the ESELp-based lentiviral system by proinflammatory cytokines. Mice were injected subcutaneously with matrigel containing MLEC infected with either LV-SFFVp-GFP or LV-ESELp-GFP, either untreated or treated with 40mg/Kg LPS (i.p.). (A) Graphic shows serum IL6 levels (mean±SD) from a representative experiment. (B) Scatter plots show in vivo luciferase activity measure (flux) of mice from a representative experiment (ESELp: p=0.0090;n=9). (C) Bioluminescence images of mice from a representative experiment. (D). Graph shows in vivo luciferase activity (flux) as fold induction of LPS-treated vs. control mice (mean± SD of three independent experiments;***p<0.001 vs untreated mice).

2.4 ESELp-driven transgene expression is modulated by proinflammatory cytokines in a murine model of chronic proliferative arthritis

We next addressed the inducibility of our lentivector system in a murine model of rheumatoid arthritis, a disease characterized by chronic inflammation of the joints which leads to cartilage and bone destruction (McInnes and Schett, 2007). Since rheumatoid arthritis typically progresses through cycles of relapse and remission, the production of a therapeutic protein must be regulated in order to match the recurrent course of the disease (Miagkov et al, 2002). We used a zymosan-induced arthritis (ZIA) model, where the intraarticular administration of zymosan induces the secretion of inflammatory interleukins (Asquith et al, 2009). An acute phase is developed during the first week after zymosan administration, which then turns into chronic inflammation (Darren et al, 2009). LV-SFFVp-Luc or LV-ESELp-Luc was injected subcutaneously in both paws one week before inducing the arthritis, to allow sufficient time for integration of the vector genome. After one week zymosan was subcutaneously injected in the right paw and physiological solution in the left paw as a negative control; as the injections are local, every animal served as its own control.

Luciferase expression was monitored by imaging of luciferase bioluminescence every three-to-four days over the first two weeks, when the signal intensity started to decrease, with additional measurements after 20 and 30 days. Compared with control paws, the arthritic paws showed a notable induction of ESELp-driven luciferase expression four days after zymosan injection, reaching a maximum after seven days (Figure 23A-C). This coincides with the time when the acute inflammation phase is known to recede (Keystone et al, 1977) and, accordingly, the luminescence signal decreased from this point. In contrast, paws injected with SFFVp-Luc showed no differences in luciferase expression upon injection with zymosan (Figure 23A-C).

Comparable progression of ZIA (zymosan-induced arthritis) in LV-SFFVp-Luc and LV-ESELp-Luc-injected paws was confirmed by measuring paw diameter (Figure 23D). In addition, inflammation was measured by bioluminescence after i.p. administration of luminol, which allows quantitative longitudinal monitoring of myeloperoxidase (MPO) system activity (Gross et al, 2009) (Figure 23E). Lentivector injection by itself produced

no inflammatory reaction that could contribute to ZIA, since luminol reactions were only observed in zymosan-treated paws.

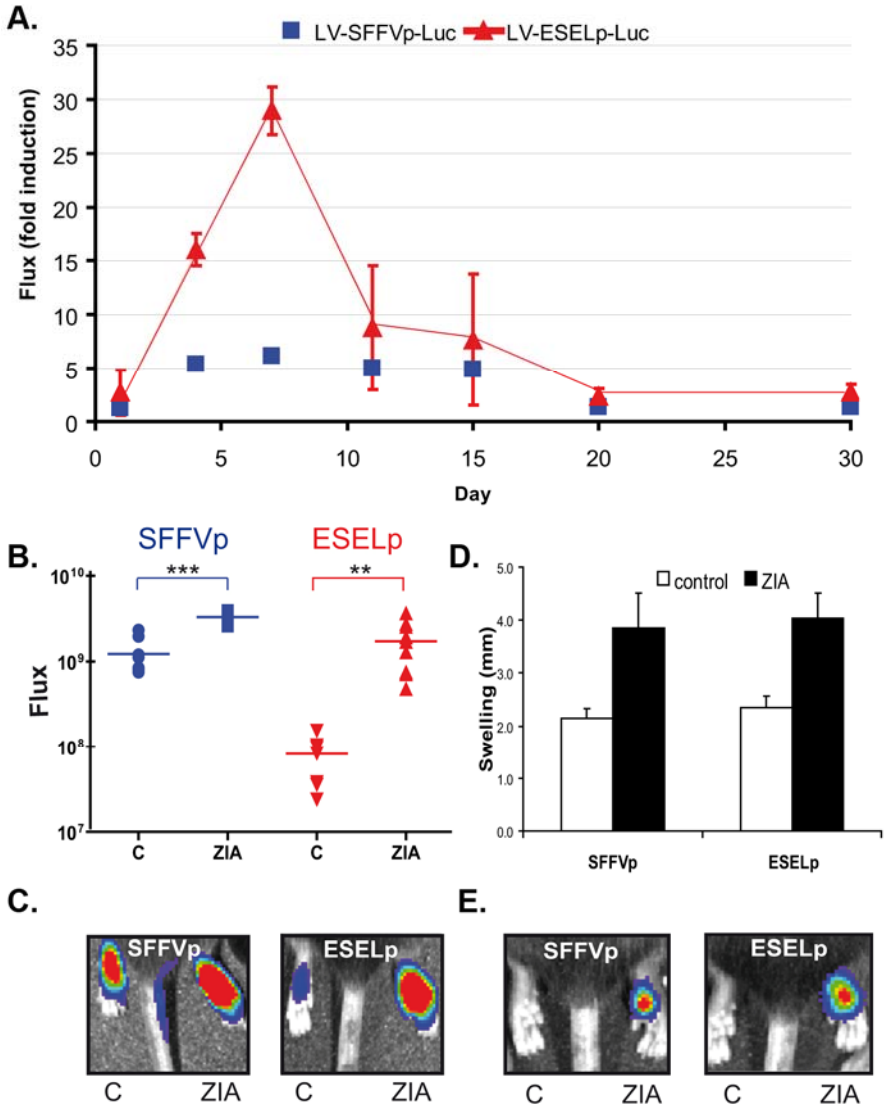

Figure 23. Local transgene expression driven by the ESELp-based lentivector at the inflammatory sites. Mice received an intra-articular injection of either LV-SFFVp-Luc or LV-ESELp-Luc in both paws. After one week, they received intra-articular injections of 180mg of zymosan in the right paw and saline solution in the left paw as a negative control for inflammation. The *in vivo* luciferase activity was determined by bioluminescence at different times after zymosan injection. (A) Graph shows the fold induction obtained from measuring the luciferase activity in both -zymosan and saline injected- paws of mice infected with LV-ESELp-Luc or LV-SFFVp-Luc at the indicated times. (B) Scatter plot shows *in vivo* luciferase activity (flux) of control and ZIA-treated paws at day 7 ($p<0.0016$; $n>7$). (C) Representative bioluminescence images from paws infected with the indicated vectors and treated with saline (-) or zymosan (+) for 7 days. (D) Graph shows the diameter (mm) of both paws of mice infected with the indicated vectors, and treated with saline (no zia) or zymosan (zia) for 7 days (mean±SD).

2.5 ESELp-driven transgene expression in vivo responds to inflammation flare-ups

An important aim in gene therapy is the development of expression systems which can be switched on and off on demand. Such vectors would allow cessation of transgene expression upon resolution of the pathological process, and its restoration should the disorder reactivate. We therefore wanted to determine whether our lentiviral ESELp-driven expression system is modulated by the inflammatory conditions induced by zymosan. We monitored the inflamed paws after the first injection of zymosan by weekly measurement of the bioluminescence produced in response to i.p. administration of luminol. After one month, no detectable bioluminescence signal was generated in the paws, and correspondingly control and zymosan-injected paws showed no differences in ESELp-driven luciferase activity (day 30, Figure 24A). At this point, we reactivated the inflammation by administering a second zymosan injection to the same paw, and monitored SFFVp- and ESELp-controlled luciferase expression by bioluminiscence.

The new inflammatory process again led to an increase in ESELp-driven transgene expression in the zymosan-injected paws, whereas no apparent changes were observed in paws infected with LV-SFFVp-Luc (Figure 24B-C). The acute inflammatory reaction induced by the second zymosan injection was similar in LV-SFFVp-Luc and LV-ESELp-Luc-infected mice, as estimated by luminol bioluminescence and paw diameter (Figure 24D-E). These data indicate that the ESELp-driven lentiviral expression system has the potential to selectively target inflammatory tissues and can be re-induced by acute inflammatory episodes.

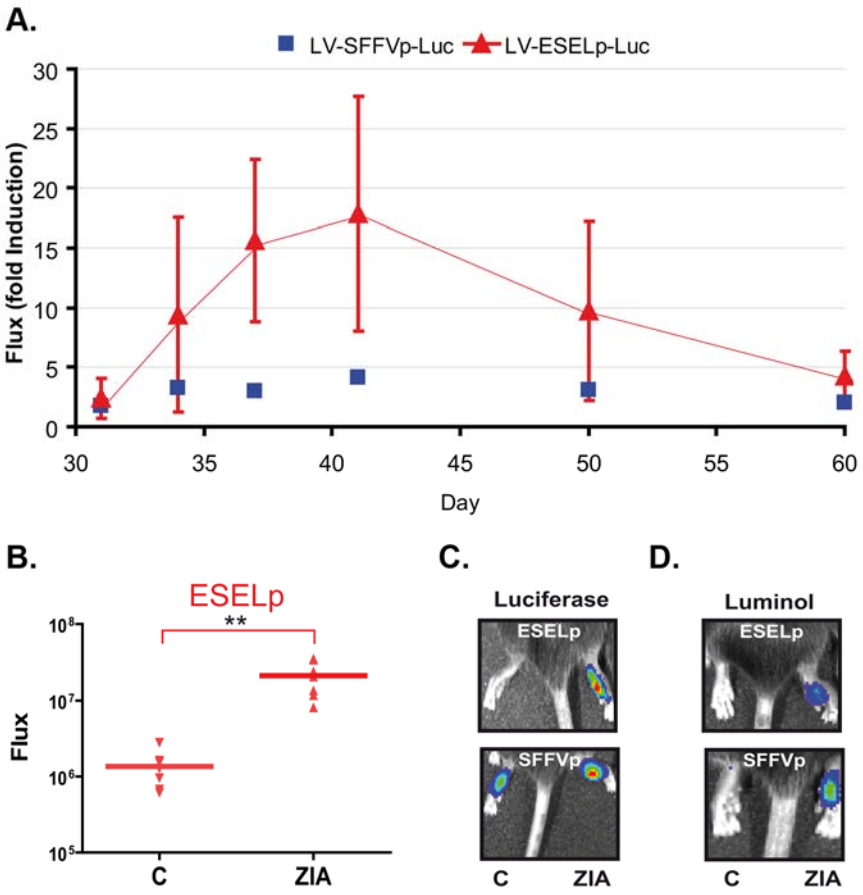

Figure 24. On/off inducibility of the ESELp-based lentiviral system in an in vivo model of arthritis. (A) One month after the first zymosan injection, when the inflammation had disappeared, mice were injected again with saline or 180mg zymosan to reactivate inflammation. Graph shows fold induction of luciferase activity measured by bioluminescence in zymosan vs. saline-reinjected paws from mice infected with LV-ESELp-Luc or LV-SFFVp-Luc at the indicated times. **(B)** Scatter plot shows *in vivo* luciferase activity (flux) of control and ZIA-treated paws 7 days after the second zymosan boost (p=0.0027; n=7). **(C,D)** Representative bioluminescence images from paws infected with the indicated vectors after the second inflammatory insult (left=luciferase; right=luminol).

Discussion

The concept that atherosclerosis is an inflammatory disease has now gained wide acceptance in the field of cardiovascular research. Substantial evidence now indicates that a wide range of cellular effectors promote atherogenesis by mediating inflammatory cascades. For this reason, we might as well think that the most straightforward treatment for such inflammatory-driven disease would consist in anti-inflammatories administration. However, many existing systemic anti-inflammatory strategies such as glucocorticoids, non-steroidal anti-inflammatory drugs, or anti-cytokine agents exert unwanted actions that render them less than ideal candidates for evaluation as long-term therapeutics for modulation of atherosclerosis (Libby et al, 2009). A growing body of evidence supports the use of statins as an anti-inflammatory intervention in atherosclerosis due to both LDL-lowering and direct anti inflammatory actions. The use of statins has been shown to provide very effective therapy for both primary and secondary prevention. Nevertheless, despite statin treatment, a large percentage of patients continues to experience cardiovascular events (Badimon and Ibañez, 2010). There is an urgent need of more specific anti-inflammatory agents for atherosclerosis, as none of the developed up to date has been sufficiently validated for clinical use. The identification of molecular mediators of inflammation that operate during atherogenesis has generated considerable interest as promising alternatives for anti-inflammatory treatments of atherosclerosis.

1. The Role of CN/NFAT Pathway in Atherosclerosis

Inflammation plays a pivotal role in host defense against infectious agents and injury, but it also contributes to the pathophysiology of many chronic diseases. Atherosclerosis provides a good and recently discovered example of a chronic inflammatory disease (Ross, 1999; Libby, 2006; Libby et al, 2009). Atherosclerosis, formerly considered a lipid storage disease in the vessels, actually involves an ongoing inflammatory response. The CN/NFAT pathway is a major signalling pathway involved in the inflammatory response, especially in T-cell activation and the regulation of cytokine gene expression in a variety of cell types (Donners et al, 2005). This pathway regulates the expression of several cytokines such as IL-1, IL-2, IFN-γ, TNF-α and CD40-ligand. Besides T-cells, CN and downstream transcription factors are also expressed in several other vascular cells such as ECs, macrophages and VSMC (Kockx et al, 2010).

Also, this pathway has been described in several other processes involved in atherosclerosis such as apoptosis (De Windt et al, 2000) and angiogenesis (Hernandez et al, 2001). NFAT is regarded as an important transcriptional regulator of cytokine and growth factor expression in T cells and cardiomyocytes. In their study, Yu and collaborators (2006) showed that NFAT also regulates cellular activation of macrophages, VSMCs, and ECs, suggesting a key role of this transcription factor in various vasculopathies such as atherosclerosis and restenosis.

We hypothesize that the transcriptional blockade of all the pro-inflammatory cytokines numbered before by inhibition of the CN/NFAT pathway could have beneficial effects in atherogenesis. To prove our hypothesis we used the well established apoE mouse model. The lesion development and plaque composition in apoE mice are similar to those in humans, establishing it as an excellent animal model for studying the pathogenesis of atherosclerosis.

As LVs have been used for long term systemic expression of genes in mice, we tested whether the CN blocking peptides were efficiently delivered to the vascular wall of the apoE mice. The present study demonstrates that LVs can be used to infect vessels after a single intravenous injection. This approach enabled our vectors to be expressed at consistent levels in the vascular wall. Due to their long term expression we were able to detect, two months after the injection, GFP expression in cross-sections of aorta and heart valves (Figure 12). These *in vivo* results were consistent with our *in vitro* results, were we tested our viral vectors driven by the ubiquitous promoter (SFFVp) and efficiently infected different cell lines and primary endothelial cells, both murine and human (Figure 18).

Additionally, we could observe the direct effect induced by the LxVP peptide-carrying vector as an inhibition in the progression of the atherosclerotic plaque formation in the aorta (Figure 14). Being all groups (except for the non-fat diet group) fed with the same high-cholesterol diet, we found a high atherosclerotic burden in the control groups (non infected and infected with GFP) which was diminished to non-fat diet levels in the groups where CN activity was inhibited (i.e., LXVP and CsA groups). Therefore, our results show that the inhibition of the CN activity, either by the

expression of the LxVP peptide or by pharmacological treatment, diminishes the atherosclerotic plaque formation.

Treatment with LxVP peptide proves to be more efficient than the VIVIT peptide. Rodriguez and collaborators (2009) recently showed that LxVP peptide interferes with the interaction of NFAT with activated CN and, unlike VIVIT, also inhibits CN phosphatase activity. Inhibition of NFAT activation by the VIVIT peptide does not reflect inhibition at the calcineurin active site, thus, the VIVIT peptide selectively inhibits NFAT activation without disrupting other CN-dependent pathways (Aramburu et al, 1999). The ability of the LxVP peptide to both strongly block the CN-NFAT interaction and the CN phosphatase activity could explain the efficient effect observed after LxVP expression in our study.

In addition to the diverse effects encountered by the different treatments, the fact that lesion size differs between the two control groups, PBS and GFP, implies an evident effect of the infection in the progress of the disease. Atherosclerotic burden significantly higher in the GFP group than in the rest of the rich fat diet fed groups, including PBS group. Endothelial dysfunction is a key event in the initiation and progression of atherosclerotic vascular disease. Endothelial activation caused by risk factors, in this case hypercholesterolemia due to rich fat diet, leads to a pro-inflammatory, proliferative and prothrombotic state of the endothelium that can accelerate the atherosclerotic process. Experimental studies demonstrate a direct impact of viral components on the vascular endothelium, as they lead to expression of adhesion molecules and pro-thrombotic factors (Barbaro, 2002). As a matter of fact, we confirmed by mRNA that in the animals treated with the viral vectors there is an increase of the expression of several pro-atherogenic genes (data not shown). This evidence supports the notion of infection/inflammation as a contributor to endothelial dysfunction that would trigger the atherogenic process in the vascular wall.

In this study we found that the beneficial effect of our treatments is more evident in the aortic arch than in the thoracic aorta. This result is strongly related to the disease susceptibility that influences the endothelium locally in an *in vivo* environment. The

spatial and temporal characteristics of blood flow are an important determinant of athero-susceptibility or athero-protection. Disturbed flow regions, such as curvatures, branches and bifurcations in arteries as well as downstream side of heart valves, correlate closely with susceptibility to the focal development of atherosclerosis, whereas adjacent undisturbed flow regions are not. Consequently, lesion-induced disturbed flow may contribute to the growth of the lesion over time. *In vitro* and *in vivo* studies of endothelial cells have demonstrated that such an environment promotes proinflammatory gene and protein expression that is conducive to increased atherosclerosis-susceptibility, plaque growth and instability, and increased risk of thrombosis (Davies, 2009). Endothelium at such locations expresses significant differences in gene expression compared with adjacent undisturbed laminar flow regions such as thoracic or abdominal aorta (Figure 25).

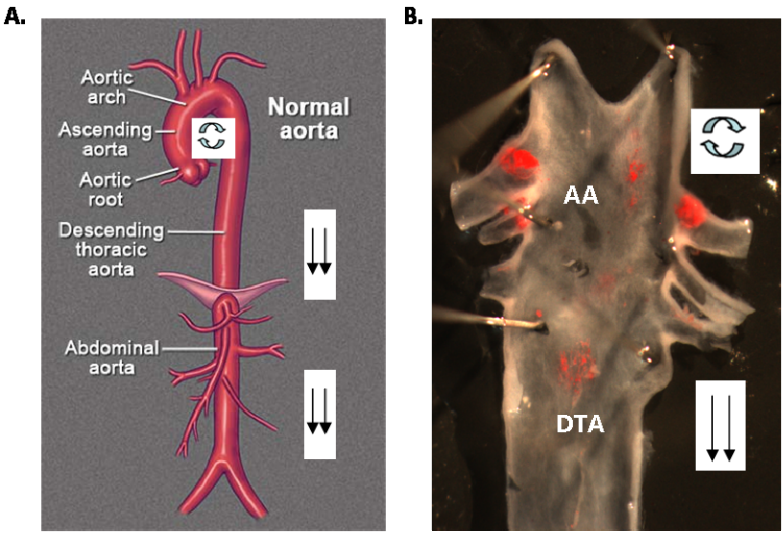

Figure 25. Effect of the diffences in the blood flow in arterial sites. Disturbed flow meaning athero-susceptibility (rotating arrows) and unidirectional shear stress implying athero-protection (straight arrows). Inner curvature of the athero-susceptible aortic arch (AA) and protected descending thoracic aorta (DTA) unopened **(A)** and **(B)** opened view (obtained from one of the animals in our study). *Adapted from Davies et al, 2010.*

In laminar flow areas such as thoracic aorta there is less susceptibility to develop atherosclerosis, so the effect of the inhibitory peptides is less evident. On the other hand, the aortic arch is much more prone to develop atherosclerotic lesions so the effect we see with our treatment is more dramatic. Nevertheless, the trend is the same in both areas besides the magnitude of the effect.

Lesions in the apoE mice, as in humans, tend to develop at vascular branch points and progress from foam cell stage to the fibroproliferative stage with well-defined fibrous caps and necrotic lipid cores (Jawien et al, 2004). After 9 weeks on high cholesterol diet, most apoE mice have developed fatty-streak lesions comprised primarily of foam cells with migrating SMCs. In the heart (aortic root and ascending aorta) we observe these fatty-streak lesions that will rapidly progress to advanced lesions, which are heterogeneous but are typically comprised of a necrotic core surrounded by proliferating smooth muscle cells and varying amounts of extracellular matrix. In this study we found no significant differences among the different experimental groups in terms of heart lesion size and composition; the different cardiac cross-sections stainings show similar neointimal amount of macrophages, VSMC and collagen (Figure 16). These lesions have progressed in every group to the formation of a lipid core with collagen and VSMC infiltration.

Preliminary evidence suggests that in the case of short duration hypercholesterolemia, paradoxical protective measures are induced in the endothelium in response to shear stress, as it is the case of the aortic valve endothelium that can develop a more marked protective adaptation than that identified in arteries (Davies et al, 2010). In our case, the efficient infection observed in the aortic root in addition to the hypercholesterolemic diet effect may have induced a temporal protective effect in the valve endothelium, slowing down the atherogenic process, which could explain the lack of a dramatic effect in the heart valves among the different treatments in our study.

2. Blocking peptides versus Immunosuppressants in Atherosclerosis

CsA and FK506 are well known immunosppressive agents for suppressing inflammation by inhibiting the activation of T-cells and are currently used to treat and prevent rejection of organ transplants, as well as several autoimmune diseases. However, long term treatment with CsA is associated with many side effects including hyperlipidemia and an increased risk of atherosclerosis. While its immunosuppressive effects are closely linked to the inhibition of T cell activation via the CN/NFAT pathway blockade, the precise mechanisms underlying its cardiovascular side effects appear to involve

multiple pathways additional to those relevant for immunosuppression. Hyperlipidemia, observed in approximately 60% of renal transplant patients, is one of the risk factors that trigger the development of atherosclerosis. Although the statin treatment helps to reduce the cardiovascular risk in these patients, the side effects of CsA remain to be a clinical important issue (Kockx et al, 2010).

However, it is not clear whether the use of CsA and FK506 either inhibits or ameliorates the development of transplant and *de novo* atherosclerosis. Previous studies on the effect of FK506 and CsA in hyperlipidemic animals were contradictory (Hamada et al, 2010; Böhmer et al, 2010). As it was already mentioned, this discrepancy may be in part due to CN-independent effects associated with both CsA and FK506. We expect to improve this situation by selectively inhibit CN/NFAT signaling with specific CN blocking peptides, VIVIT and LxVP, which directly interact with CN and block the CN/NFAT signaling pathway. We tested the hypothesis that the expression of these peptides would attenuate atherosclerotic plaque development in apoE mice on a high-fat diet. To test this hypothesis, we attempted to inhibit atherogenesis in its early stages by systemic injection of an LV which expressed either inhibitory peptide, VIVIT or LxVP, and compared their effects with CsA-treated animals. We found that the inhibition of CN signaling with LxVP peptide is more efficient than with VIVIT, and that its effect, along with the CsA-treated group, lowers the lesion size to levels of the normal diet group, both in aortic arch and thoracic aorta. VIVIT peptide follows this trend but the difference with the normal diet group is not statistically significant in either of the two areas. This could be due to the difference in the interaction with CN between both peptides. Whereas VIVIT specifically interrupts CN docking onto NFAT allowing efficient NFAT inactivation without impairing CN phosphatase activity, LxVP not only prevents CN-NFAT interaction, but also inhibits CN phosphatase activity. For this inhibition, LxVP binds in a site between the two subunits of CN, preventing its activity. This binding site could be shared by LxVP and the immunosuppressants CsA and FK-506 as they all have a similar effect, although LxVP has not known side effects. This results would imply that the solely blockade of CN-NFAT interaction produced by VIVIT is not as effective as the inhibition of CN activity in general to attenuate the progression of atherosclerosis by a high fat diet. These results

demonstrate that blocking the CN activity hinders the development of atheroma plaques in the aorta of apoE mice. Therefore they support the hypothesis that CN plays an important role in atherosclerosis and that its inhibition in the early stages of the disease has athero-protective effects.

3. LxVP as an immunosuppressive peptide

The most common method for blocking CN-NFAT pathway is the administration of CsA or FK506, which inhibits the enzymatic activity of CN. As it was mentioned earlier, CsA and FK506 must bind to a specific IP for the CN activity inhibition (Cardenas et al, 1995; Liu et al., 1991). Although CsA (a cyclic peptide) and FK506 (a macrolide) are structurally unrelated, they interact with the same residues in a common site in CN suggesting that this site is involved in the recognition of other CN-binding proteins. In fact, these complexes compete with LxVP for CN, suggesting that they bind to the same docking surface on CN. In contrast with CsA and FK506, LxVP peptide interacts directly with CN and it would be expected that this peptide exclusively affects CN phosphatase activity. Administration of the LxVP peptide would therefore be expected to have fewer side-effects than treatments with CsA or FK506. Moreover, the LxVP peptide could be a useful tool for identifying which CsA and FK506 side effects are unrelated to the inhibition of CN phosphatase activity which may contribute to the adverse effects associated with the clinical use of these drugs.

Despite the results obtained by Emeson et al (1993) where they demonstrated larger atherosclerotic plaques in CsA-treated animals than in the control group, the dose of CsA used and the duration of the treatment were satisfactory in our study, as we also obtained inhibition of the plaque formation in the CsA group. However, some renal damage was already observed in CsA-treated animals, whereas this damage was not observed in cross-sections from the kidneys of the viral-treated and control groups (Figure 17). As the favorable effects obtained with the LxVP blocking peptide were similar to those obtained with CsA, they suggest that selective blockade of CN with agents more specific than the current immunosuppressive drugs may be useful for the treatment of atherosclerosis. These results encourage the development of new CN

blocking agents mimicking these peptides; such agents would avoid some side effects associated with the FK506 and CsA.

4. An Inflammation-regulated Viral Vector based on the E-selectin Promoter

The inflammatory response is precisely controlled by the expression of cytokines whose local levels are directly related to the severity of the process. A major challenge in the treatment of chronic inflammatory diseases is the development of an expression system tightly regulated by the variable levels of these cytokines. In this work, we describe a long-term lentiviral expression system based on the E-selectin promoter, which is locally induced by inflammatory stimuli in direct relationship to the intensity and duration of the inflammatory response.

Studies in animal models have shown that gene therapy is an alternative for the local treatment of chronic inflammatory diseases. One of the critical factors in gene transfer is the type of vector employed. Non-viral vectors commonly yield low gene transfer efficiency (Li and Huang, 2000; Niidome and Huang, 2002). Among the viral vectors, adenoviruses are the most widely used; however, they are not good candidates for the treatment of chronic inflammatory diseases due to the immune response associated with their application and the rapid loss of transgene expression due to lack of persistence of the viral genomes (Newman et al 1995, Hiltunen et al 2000). First-generation adenoviral (FGAd) vectors efficiently transduce EC both in vitro and in vivo. However, numerous *in vitro* studies show that FGAd can alter cellular proliferation, migration, and apoptosis, and increase expression of inflammatory cytokines and adhesion molecules (Flynn et al, 2010).

Adeno-associated viruses (AAV) have emerged as a very promising tool for gene transfer in chronic inflammatory diseases. Although AAV vectors have limited cargo capacity, they stably transduce host cells and show low immunogenicity. Vectors based on the human-derived adeno-associated virus serotype 2 (AAV2) have achieved stable transgene expression in a number of preclinical models targeting a wide variety of organs such as liver, skeletal and cardiac muscle, retina and the central nervous system (Lebherz et al, 2004). However, recent works have reported an inflammatory response

after AAV application (Mingozzi and High, 2007; Zaiss et al, 2008; Peden et al, 2009); in addition, their scalability to produce adequate viral titers is hampered by technical limitations (Pajusola et al, 2002; Eslami et al, 2000; Vassalli et al, 2003).

As an alternative, lentivirus-derived expression systems have been employed in neuroinflammation animal models (van Strien et al, 2010). Our study suggests that lentivectors (LVs) may be an alternative in the treatment of chronic inflammatory diseases; LVs not only infect dividing and quiescent cells, but they also provide long-term expression and show low immunogenicity. In addition, the biosafety profile of lentiviral vectors has been improved significantly by minimizing the regions of homology between vector and helper sequences (split configuration), and by using heterologous promoters (Vandendriessche et al, 2002). Furthermore, the use of the Vesicular Stomatitis Virus Glycoprotein (VSV-G) confers efficient transduction in a wide range of cell types from many species, and allows the concentration of the lentiviral particles (Burns et al, 1993, Bartz et al, 1996).

Several inflammation-inducible systems have been recently described, all of which are based on chimeric promoters. These promoters have been artificially tailored and their precise in vivo regulation is still unknown. Our expression system is based on the proximal promoter region which controls the expression of the E-selectin gene. This gene is particularly attractive as it is induced early and transiently upon inflammation and its promoter region contains the binding sites for transcription factors induced by the early-induced pro-inflammatory cytokines, TNF-α and IL-1. We have tested our expression system in an experimental model of chronic joint inflammation administering repetitive local injections of zymosan (heat inactivated yeast). We found that E-Selectin promoter (ESELp) is rapidly induced after zymosan administration along with the peak of inflammation, 7 days after the first treatment, and that transgene expression is maintained until inflammation recedes. Our results show that transgene expression increases several fold which correlates with the severity of the inflammation. As chronic inflammatory diseases are characterized by flare-ups and remission phases, it was important to test whether the promoter was not silenced in vivo and that transgene expression could be re-induced after a second zymosan boost.

We observed again a correlation between transgene expression and inflammatory status after the second zymosan boost, showing no evidence of promoter silencing. Therefore, we consider the ESELp a valuable tool to develop gene expression systems potentially useful for the treatment of chronic inflammatory diseases. Selective and local expression of anti-inflammatory agents in arthritis-affected joints employing ESELp-based gene delivery systems might eliminate some of the problems encountered with systemic drug therapies, such as tolerability and compliance.

Treatment of chronic inflammatory diseases is complicated as they are characterized by unpredictable relapses. Rheumatoid arthritis (RA), the most frequent inflammatory rheumatic disorder, is a prototype of chronic inflammatory disease characterized by an imbalance of pro- and anti-inflammatory molecules. Although systemic administration of anti-inflammatory agents is beneficial to patients with long-lasting RA, these treatments are limited by loss of efficiency and relapse after stopping the treatment. There are also important side effects associated with the prolonged systemic counterbalance to the inflammatory response (Bongartz et al 2006, Scott et al 2006). Viral vectors are promising candidates for gene therapy for local treatment of RA; in fact, they are currently employed in several clinical trials of RA gene therapy (Chan et al 2002). However, there is still a need to develop new therapeutic approaches that provide prolonged remission from disease with limited side effects by targeting anti-inflammatory mediators to the diseased joints. The use of disease-regulated promoters to drive transgene expression might provide therapeutic levels of the anti-inflammatory agent exclusively during flares. In addition, local administration into arthritic joints should minimize the side effects resulting from systemic administration and increase the site-specific effects of the therapeutic agent.

We demonstrate that intra-articular delivery of our lentiviral expression system is regulated by the local inflammation level. We show that the transgene expression correlates with the progression of the arthritis in the animal model tested. As RA is characterized by cycles of relapse and remission, we re-induced the inflammation after the first remission and found that the transgene was again transiently up-regulated. As expected, the inflammation level and transgene induction were lower than that

observed at disease onset. Therefore, since our expression system is endogenously regulated, it could represent a new approach for local treatment of chronic inflammatory diseases due to its disease-regulated expression of anti-inflammatory molecules.

Conclusions

The conclusions drawn from this work can be resumed as follows:

1. Blocking CN activity by either CsA treatment or expressing the LxVP peptide attenuates atherosclerosis progression in the aorta of apoE mice on a high fat diet. These results suggest that CN activity contributes to plaque development in early stages of the disease. Therefore, inhibition of CN activity, by employing either CN inhibitory peptides or newly developed drugs, stands as promising treatment or co-treatment to delay plaque development in high risk patients.

2. Since the expression of the VIVIT peptide, which selectively blocks the CN-NFAT interaction, did not attenuate the atheroma burden, CN substrates besides NFAT must be involved in the plaque formation in the aorta of apoE mice on a high-fat diet.

3. The lentiviral system based on the E-selectin promoter responds to inflammatory stimuli *in vitro* and *in vivo*, suggesting their therapeutic application in the treatment of chronic inflammatory diseases.

4. Intra-jugular administration of lentiviral particles is an efficient method for vascular wall transduction in aorta and heart that may represent an alternative route for the efficient delivery of therapeutic molecules to the cardiovascular system.

Conclusiones

Las conclusiones extraídas de este trabajo son las siguientes:

1. El bloqueo de la actividad de CN mediante el tratamiento con CsA o mediante la expresión del péptido LxVP atenúa la progresión de la aterosclerosis en aorta de ratones apoE alimentados con una dieta alta en grasas. Estos resultados sugieren que la actividad CN contribuye al desarrollo de placa en las primeras etapas de la enfermedad. Por lo tanto, la inhibición de la actividad CN, mediante el empleo de péptidos inhibidores o de nuevos fármacos inhibidores, supondría un posible tratamiento o co-tratamiento para retrasar el desarrollo de placas de ateroma en pacientes de alto riesgo.

2. Dado que la expresión del péptido VIVIT, que bloquea selectivamente la interacción CN-NFAT, no afecta al desarrollo de las placas de ateroma, otros sustratos de CN además de NFAT deben participar en la formación de las placas de ateroma en la aorta de ratones apoE alimentados con una dieta alta en grasas.

3. El sistema lentiviral basado en el promotor de la E-selectina responde a estímulos inflamatorios tanto *in vitro* como *in vivo*, sugiriendo su posible aplicación terapéutica en el tratamiento de enfermedades inflamatorias crónicas.

4. La administración intra-yugular de las partículas lentivirales es un método eficiente para la transducción de la pared vascular en aorta y corazón, puediendo representar una ruta alternativa para la administración eficiente de moléculas terapéuticas en el sistema cardiovascular.

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